(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 September 2000 (14.09.2000)

PCT

(10) International Publication Number
WO 00/053625 A3

(51) International Patent Classification[7]: C07K 1/04, G01N 33/68, 33/543, B01J 19/00

(21) International Application Number: PCT/US00/06676

(22) International Filing Date: 10 March 2000 (10.03.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/123,877 11 March 1999 (11.03.1999) US
60/123,791 11 March 1999 (11.03.1999) US

(71) Applicant *(for all designated States except US)*: **COMBIMATRIX CORPORATION** [US/US]; 34935 S.E. Douglas Street, #110, Snoqualmie, WA 98065 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **ROSSI, Francis, M.** [US/US]; 303 Robin Way, Menlo Park, CA 94025 (US). **MONTGOMERY, Donald, D.** [US/US]; 1410 Millbrae Avenue #306, Millbrae, CA 94030 (US).

(74) Agent: **HALLUIN, Albert, P.**; Howrey Simon Arnold & White, LLP, Box 34, 1299 Pennsylvania Avenue, N.W., Washington, DC 20004 (US).

(81) Designated States *(national)*: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

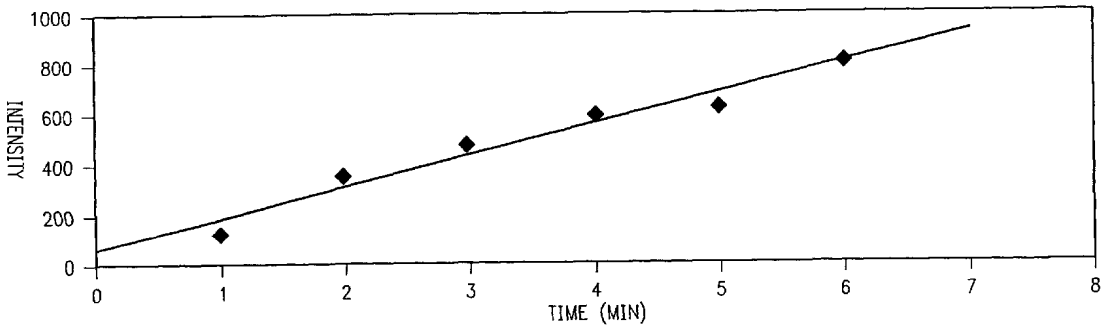
(88) Date of publication of the international search report:
5 April 2001

(48) Date of publication of this corrected version:
4 July 2002

(15) Information about Correction:
see PCT Gazette No. 27/2002 of 4 July 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: MICROARRAYS OF PEPTIDE AFFINITY PROBES FOR ANALYZING GENE PRODUCTS AND METHODS FOR ANALYZING GENE PRODUCTS



(57) Abstract: The present invention features an array of peptide probes for analyzing gene products. Preferably, the array of peptide probes is formed on a porous membrane proximate at least one electrode. The present invention also features a method for determining the presence of a gene product in a sample. The method features contacting a biological sample with a peptide array. Preferably, the peptide array comprises at least about 100 peptide probes. Especially preferably, the peptide array comprises at least about 1000 peptide probes.

WO 00/053625 A3

MICROARRAYS OF PEPTIDE AFFINITY PROBES FOR ANALYZING GENE PRODUCTS AND METHODS FOR ANALYZING GENE PRODUCTS

FIELD OF THE INVENTION

The present invention is in the field of biological and chemical synthesis and processing. The present invention relates to arrays and methods for analyzing gene products.

The present application claims priority to U.S. Provisional Application Serial Nos. 60/123,877 and 60/123,791 filed March 11, 1999.

BACKGROUND OF THE INVENTION

Although it is anticipated that the sequence of the human genome will be completed soon (Collins *et al. Science* 282:682-689 (1998)), the use of the resulting genetic information to identify and treat human disease will take many years. At a fundamental level, any method used to identify the cause of a disease begins with the comparison of healthy and sick patients. The comparison of the DNA sequence of healthy and sick individuals has led to the identification of many genes correlated with particular disease states. Most human disease, however, is not caused by mutations in a single gene, but rather results from the complex interactions between the products of gene expression, the proteins (Collins *et al. Science* 282:682-689 (1998); Anderson et al., *Electrophoresis* 19:1853-1861 (1988)). The identification and characterization of the gene products and the elucidation of the mechanisms by which they interact to cause disease remains a difficult scientific problem.

Any given cell contains thousands of gene products, often called the proteome. The proteome is most efficiently studied when the amounts of all of the expressed proteins can be quantitated simultaneously. One approach to this problem has been to use microarrays of DNA oligonucleotides to indirectly observe protein expression (Watson et al., *Current Opinion in Biotechnology* 9:609-614 (1998); Braxton et al., *Current Opinion in Biotechnology* (1998)). By examining the interaction of cDNA, made by reverse transcription of expressed RNA, with thousands of immobilized DNA oligonucleotide probes, these devices have been used to indirectly compare RNA expression levels between cells. Unfortunately the correlation between RNA expression levels and cellular protein levels is not strong (correlations have been shown to be less than 0.50) (Anderson et al., *Electrophoresis* 18:533-537 (1997)) and many important biological phenomena, such as signal protein phosphorylation, occur when proteins modify other proteins and thus cannot be observed by monitoring changes in the expression levels of RNA (Haynes et al., *Electrophoresis* 19:1862-1871 (1998)). Therefore, it is highly desirable to have a method for direct, simultaneous measurement of protein expression and protein interactions.

Any useful system for the direct analysis of gene products has two essential characteristics; (1) the ability to separate a complex mixture of thousands of proteins, and (2) the

Any useful system for the direct analysis of gene products has two essential characteristics; (1) the ability to separate a complex mixture of thousands of proteins, and (2) the ability to assay quantitatively the separated proteins. The most commonly used separation method, 2-dimentional gel electrophoresis (Jungblut *et al., Electrophoresis* 17:839-847 (1996)), separates proteins by isoelectric point and molecular weight. The proteins are then visualized by staining, usually with silver. Although the reliability of 2D-electrophoresis has improved considerably in recent years, the technique suffers from limitations that include complex signal patterns that must be analyzed by sophisticated computer algorithms, and the inability to resolve hydrophobic, large, or basic proteins (Wilkins, *Electrophoresis* 17:830-838 (1996)). In addition, signals from 2-D electrophoresis are difficult to quantitate since the susceptibility of individual proteins to stain varies. One way to compensate for this phenomena is to compare the experimental sample with an internal control that is stained with a second dye. As long as both dyes modify proteins by the same chemical mechanism, the experimental and control sample will be stained to the same extent. Unfortunately, samples separated by two-dimentional gel electrophoresis cannot be stained before the isoelectric focusing step, (Urwin et al., *Anal. Biochem.* 195:1-37 (1991)) and thus it is not possible to run two samples on the same gel and compare the relative protein expression levels by two-color staining techniques.

Methods of preparing large numbers of different ligands have been painstakingly slow and prohibitively expensive when used at a scale sufficient to permit effective rational or random screening. For example, the method described by Merrifield et al, *J. Am. Chem. Soc.* 85:2149-2154 (1963) has been used to synthesize peptides on solid supports. In this method, an amino acid is bound covalently to a support made of an insoluble polymer. Another amino acid with an alpha protected group is reacted with the covalently bonded amino acid to form a dipeptide. After washing, the protective group is removed and a third amino acid with an alpha protective group is added to the dipeptide. This process is continued until a peptide of a desired length and sequence is obtained. Using the Merrifield method, synthesis of more than a handful of peptide sequences in a day is not technically feasible or economically practical.

To synthesize larger numbers of polymer sequences, it has been proposed to use a series of reaction vessels for polymer synthesis. For example, a tubular reactor system may be used to synthesize a linear polymer on a solid phase support by automated sequential addition of reagents. This method, however, also does not enable the synthesis of a sufficiently large number of polymer sequences for effective and economical screening.

Another method of preparing a plurality of polymer sequences uses a porous container enclosing a known quantity of reactive particles, larger in size than pores of the container. The

particles in the containers may be selectively reacted with desired materials to synthesize desired sequences of product molecules. However, as with the other methods known in the art, this method is not practical for the synthesis of a sufficient variety of polypeptides for effective screening.

Other techniques have also been described and attempted. Several of these methods include synthesis of peptides on 96 plastic pins that fit the format of standard microtiter plates. Unfortunately, while these techniques have been somewhat useful, substantial problems remain. Methods using standard microtiter plates continue to be limited in the diversity of sequences that can be synthesized and screened. Although it is recognized that using microtiter plates produces essentially pure polymers because each polymer is synthesized in an isolated well of the microtiter plate, the number of polymers that can be produced in any given time is limited by the number of wells in a microtiter plate, *i.e.*, 96. Moreover, the equipment needed for synthesis in the microtiter plates is large. Because of this limitation, use of microtiter plates requires a large amount of space to produce a relatively small number of peptides.

One attempt at synthesizing a large number of diverse arrays of polypeptides and polymers in a smaller space is found in U.S. Patent No. 5,143,854 granted to Pirrung et al. (1992). This patent describes the use of photolithographic techniques for the solid phase synthesis of arrays of polypeptides and polymers. The disclosed technique uses "photomasks" and photolabile protecting groups for protecting the underlying functional group. Each step of the process requires the use of a different photomask to control which regions are exposed to light and thus deprotected. The necessity of having to fabricate a new set of photomasks for each array of chemical monomers results in a method that is extremely expensive and not well-suited to automation. Moreover, this method is tedious and time consuming because each step of the synthesis requires the mechanical removal, replacement and realignment of a photomask. Thus, synthesizing a large number of libraries of polymers with the Pirrung method is an inefficient and uneconomical process.

Another drawback of the Pirrung method is that the photolabile protecting groups used cannot be removed as effectively as conventional acid or base labile protecting groups can be removed and are plagued by contamination due to undesired side reactions. Consequently, using Pirrung's method, the purity of the chemical array is often compromised due to incomplete removal of the protecting groups and subsequent failure of the underlying functional groups to react with the desired monomer, as well as contamination from undesired side reactions.

Another attempt to synthesize large numbers of polymers is disclosed by Southern in International patent application WO 93/22480, published November 11, 1993. Southern describes

a method for synthesizing polymers at selected sites by electrochemically modifying a surface; this method involves providing an electrolyte overlaying the surface and an array of electrodes adjacent to the surface. In each step of Southern's synthesis process, an array of electrodes is mechanically placed adjacent the points of synthesis, and a voltage is applied that is sufficient to produce electrochemical reagents at the electrode. The electrochemical reagents are deposited on the surface themselves or are allowed to react with another species, found either in the electrolyte or on the surface, in order to deposit or to modify a substance at the desired points of synthesis. The array of electrodes is then mechanically removed and the surface is subsequently contacted with selected monomers. For subsequent reactions, the array of electrodes is again mechanically placed adjacent the surface and a subsequent set of selected electrodes activated.

This method requires that a large amount of control be exercised over the distance that exists between the electrode array and the surface where synthesis occurs. Control over the distance between the electrodes and the surface for modification is required to ensure precise alignment between the electrodes and the points of synthesis and to limit the extent of diffusion of electrochemically generated reagents away from the desired points of synthesis. However, the inherent difficulty in positioning electrodes repeatedly and accurately within a few microns of the surface frequently results in the production of electrochemically generated reagents at undesirable synthesis points. Moreover, the diffusion of the electrochemically generated reagents from desired sites of reaction to undesired sites of reaction results in "chemical cross-talk" between synthesis sites. This cross-talk severely compromises the purity of the final product, as undesired binding reactions occur at unselected sites. The amount of cross-talk is further aggravated by the disruptions of surface tension that occur whenever the electrodes are moved, leading to convective mixing that causes increased movement of the electrochemically generated reagents. While Southern attempts to minimize the amount of cross-talk by applying a potential designed to counteract diffusion, as a practical matter, the electric fields Southern can generate are too low to prevent diffusion. When the potential is raised to increase the electric field, large quantities of undesired electrochemically generated reagents are produced. Hence, Southern is not a practical method for generating large numbers of pure polymers.

A more recent attempt to automate the synthesis of polymers is disclosed by Heller in International patent application WO 95/12808, published May 11, 1995. Heller describes a self-addressable, self-assembling microelectronic system that can carry out controlled multi-step reactions in microscopic environments, including biopolymer synthesis of oligonucleotides and peptides. The Heller method employs free field electrophoresis to transport analytes or reactants to selected micro-locations where they are effectively concentrated and reacted with the specific

binding entities. Each micro-location of the Heller device has a derivatized surface for the covalent attachment of specific binding entities, which includes an attachment layer, a permeation layer, and an underlying direct current micro-electrode. The presence of the permeation layer prevents any electrochemically generated reagents from interacting with or binding to either the points of synthesis or to reagents that are electrophoretically transported to each synthesis site. Thus, all synthesis is due to reagents that are electrophoretically transported to each site of synthesis.

The Heller method, however, is severely limited by the use of electrophoretic transport. First, electrophoretic transport requires that the reactants be charged in order to be affected by the electric fields; however, conventional reactants of interest for combinatorial chemistry are usually uncharged molecules not useable in an electrophoretic system. Second, the Heller method does not, and cannot, address the large amount of chemical crosstalk that inherently occurs because of the spatial distribution of the electric fields involved in the electrophoretic transport of the reagents for binding. In a system utilizing electrophoresis, one cannot use protecting groups to protect the reactive functional groups at the microlocations since there is no mechanism for removing the protective groups; yet, the use of electrophoresis results in various binding entities and/or reactants being located throughout the solution used as they migrate, often coming into contact with unselected reaction sites. Thus, the combination of the lack of protecting groups and the spatial distribution of the electric fields inherent to electrophoresis allow such binding reactions to occur randomly, compromising the fidelity of any polymer being synthesized.

SUMMARY OF THE INVENTION

In a first aspect, the present invention features an array of peptide probes for analyzing gene products. Preferably, the array of peptide probes is formed on a porous membrane proximate at least one electrode. Preferably, the electrode current or potential is controlled and operated by interface with a computer either proximate or remote from the electrode. In preferred embodiments, the array of peptide probes is formed on a porous or non-solid support having a plurality of electrodes proximate the porous membrane. In especially preferred embodiments, at least about 100 electrodes and peptides are present on the array. In even more preferred embodiments at least about 500 or at least about 1000 electrodes and peptides are present on the array.

In a second aspect, the present invention features a method for determining the presence of a gene product in a sample. The method features contacting a biological sample with a peptide array. Preferably, the peptide array comprises at least about 100 peptide probes. Especially preferably, the peptide array comprises at least about 1000 peptide probes. The method is

preferably performed under conditions suitable to allow binding of gene products to corresponding ligands or binding domains. Preferably, the detection is performed using fluorescently labeled tags that may be detected by such devices as epifluorescent microscopes and a CCD camera.

In a third aspect, the present invention features a method for quantifying the amount of a gene product in a sample. The method features contacting a biological sample with a peptide array. Preferably, the peptide array comprises at least about peptide probes. Especially preferably, the peptide array comprises at least about 1000 peptide probes. The method is preferably performed under conditions suitable to allow binding of gene products to corresponding ligands or binding domains.

In a fourth aspect, the present invention features a method for identifying the functional binding domain of a gene product in a sample. The method features contacting a biological sample with a peptide array. Preferably, the peptide array comprises at least about peptide probes. Especially preferably, the peptide array comprises at least about 1000 peptide probes. The method is preferably performed under conditions suitable to allow binding of gene products to corresponding ligands or binding domains.

In a fifth aspect, the present invention features a method for producing an array of peptide probes for analyzing gene products. Preferably, the array of peptide probes is formed on a porous membrane proximate at least one electrode. Preferably, the electrode current or potential is controlled and operated by interface with a computer either proximate or remote from the electrode. In preferred embodiments, the array of peptide probes is formed on a porous or non-solid support having a plurality of electrodes proximate the porous membrane. In especially preferred embodiments, at least about 100 electrodes and peptides are present on the array. In even more preferred embodiments at least about 500 or at least about 1000 electrodes and peptides are present on the array.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 describes how an array according to the present invention may be used to synthesize a pattern of molecules. First, the array may be coated with a biocompatible porous membrane that allows molecules to flow freely between a bulk solvent and an electrode. The array may then be immersed in a solution containing a precursor to an electrochemically-generated (ECG) reagent of interest. For peptide synthesis, this is preferably an ECG-reagent to remove amino protecting groups. A computer may then interface with the array to turn on the desired electrode pattern, and the precursor may be electrochemically converted into an active

species. The electrochemically-generated (ECG) reagent, in turn, reacts with molecules immobilized to the membrane overlying the electrode.

Figure 2 illustrates a central feature of preferred arrays according to the present invention. The arrays have the ability to confine electrochemically generated reagents to a region immediately adjacent to a selected microelectrode. Here, a fluorescein dye has been immobilized covalently at individually addressed microelectrode locations. The dye may be tightly confined to a checkerboard pattern and exhibits substantially no chemical crosstalk between active and inactive microelectrodes.

Figure 3 demonstrates the methods according to the present invention whereby gene products may be separated by their binding affinity to a spatially defined microarray of small peptide probes. First, an array of peptide probes is synthesized. The probe set is designed to be sufficiently large and diverse that proteins bind selectively to individual peptide probes. The peptide array is then contacted with samples of interest. To compare the protein expression levels in two samples, each sample is preferably prederivatized with a different fluorescent dye. The samples are mixed and contacted to the array. The bound proteins are visualized by fluorescence microscopy, and the image captured by a suitable device such as a CCD camera.

Figure 4 illustrates a central feature of preferred arrays according to the present invention having the ability to confine the electrochemically generated reagents to a region immediately adjacent to a selected microelectrode. Here, a fluorescein dye has been immobilized covalently at individually addressed microelectrode locations. The dye may be tightly confined to a checkerboard pattern and exhibits substantially no chemical crosstalk between active and inactive microelectrodes. This level of localization of ECG reagents may be achieved by exploiting the physical chemistry of the solution in which the microelectrode array is immersed. Such solutions usually contain buffers and scavengers that react with ECG reagents. However, the rate at which ECG reagents are produced can overwhelm the ability of the solution to react with them in the small local area immediately proximate to the microelectrode. As a result, chemistry that is mediated by ECG reagents occurs near selected microelectrodes, but there is no chemical crosstalk.

Figure 5 depicts methods for preparing a molecular array on a porous electrochemical array. The method begins by coating the array with a porous reaction layer in which the molecules of interest are synthesized. The array is then immersed in a reaction solution that is inert until it is electrochemically activated. Electrodes are turned on under the areas of the solid support where chemistry is to occur. The electrode activates the solution (either by oxidation or reduction) and produces a reagent that diffuses into the reaction layer. By formulating the

solution with an appropriate buffer, diffusion of the reagent beyond the area immediately over the electrode is eliminated. Thus, the reagent is contained in a "virtual flask" without physical walls. After the required reaction time has elapsed, the electrodes are turned off, leaving behind the chemically modified solid support.

Figure 6 depicts the structure of an especially preferred Fmoc linker according to the present invention.

Figure 7 shows how azobeneze enhances the deprotection of Fmoc-groups when compared to solvent, especially at lower voltages.

Figure 8 depicts the site specific deprotection of Fmoc-groups performed by immersing an array in a solution azobenzene. Cathodic reduction of the reagent produces base over the electrode. Standard solid-phase peptide synthesis techniques are used to produce the amide bond.

Figure 9 depicts the synthesis protocol results. The array was immersed in a solution of an activated Fmoc-protected amino acid and electrolyte. The rate of amide bond formation is enhanced by turning on the electrode. A Fmoc-protected amine was immobilized in a checkerboard pattern over the top four rows of the array. After the Fmoc-groups were removed by treating the chip with piperidine, amide bond formation between the resulting amines and Fmoc-protected glycine was electrochemically induced over the right hand column of three electrodes. The array was then treated with an amino reactive dye (anthracene dialdehyde) and imaged under an epifluorecent microscope. The dark upper right hand corner indicates that reactive amino groups are no longer present. Amide bond formation is followed by standard Fmoc-deprotection with piperidine.

Figure 10 depicts synthesis of leu-enkephalin. The Fmoc-protected tetrapeptide Fmoc-GGFL was activated and immobilized electrochemically on the array. The Fmoc-groups were globally removed by treating the array with piperidine. Leu-enkephalin was synthesized at the desired locations by electrochemically promoted amide bond formation with Fmoc-protected tyrosine.

Figure 11 depicts recognition of leu-ekephalin. The array was immersed in a buffer solution containing monoclonal mouse anti-leu-enkephalin, followed by Texas-Red labeled goat anti-mouse. The secondary antibody was detected using epifluorescent microscopy. Antibody binding to the desired peptide was ~3 fold greater than binding to tyrosine, and ~5 fold greater than binding to the tetrapeptide GGFL.

Figure 12 represents a dilution series of a Fmoc protected amino linker, on top of which peptides can be synthesized. After immobilization of the linker, the Fmoc-group was removed by treatment with piperidine, the resulting amino groups were fluorescently stained, and the chip was

imaged under an epifluorescent microscope. The intensity of each spot was measured and plotted verses the amount of time the electrode was turned on.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the present invention features an array of peptide probes for analyzing gene products. Preferably, the array of peptide probes is formed on a porous membrane proximate at least one electrode. Preferably, the electrode current or potential is controlled and operated by interface with a computer either proximate or remote from the electrode. In preferred embodiments, the array of peptide probes is formed on a porous or non-solid support having a plurality of electrodes proximate the porous membrane. In especially preferred embodiments, at least about 100 electrodes and peptides are present on the array. In even more preferred embodiments at least about 500 or at least about 1000 electrodes and peptides are present on the array. The peptide probes may be of any length desired, however, especially preferred peptide probes are about 4 to about 20 amino acids long.

The arrays of the present invention are preferably similar to those described in United States Serial Nos. 09/003,075 and 09/214,348, the disclosures of which are herein incorporated by reference. Such arrays are designed to allow synthesizing chemical compounds such as peptides at well-defined and individually addressable locations. Such arrays may be manufactured at low cost by contract fabricators using existing semiconductor manufacturing facilities. **Figure 1** describes how such an array may be used to synthesize a pattern of molecules. First, the array may be coated with a biocompatible porous membrane that allows molecules to flow freely between a bulk solvent and an electrode. The array may then be immersed in a solution containing a precursor to an electrochemically-generated (ECG) reagent of interest. For peptide synthesis, this is preferably an ECG-reagent to remove amino protecting groups. A computer may then interface with the array to turn on the desired electrode pattern, and the precursor may be electrochemically converted into an active species. The electrochemically-generated (ECG) reagent, in turn, reacts with molecules immobilized to the membrane overlying the electrode.

A central feature of preferred arrays according to the present invention is the ability to confine the ECG reagents to a region immediately adjacent to a selected microelectrode. This is illustrated in **Figure 2**. Here, a fluorescein dye has been immobilized covalently at individually addressed microelectrode locations. The dye may be tightly confined to a checkerboard pattern and exhibits substantially no chemical crosstalk between active and inactive microelectrodes. This level of localization of ECG reagents may be achieved by exploiting the physical chemistry of the solution in which the microelectrode array is immersed. Such solutions usually contain buffers and scavengers that react with ECG reagents. However, the rate at which ECG reagents

are produced can overwhelm the ability of the solution to react with them in the small local area immediately proximate to the microelectrode. As a result, chemistry that is mediated by ECG reagents occurs near selected microelectrodes, but there is no chemical crosstalk.

In some embodiments, the surface of the array may be provided with a layer of linker molecules. Linker molecules allow for indirect attachment of monomers or pre-formed molecules to the substrate or a layer overlaying the substrate. The linker molecules are preferably attached to an overlaying layer via silicon-carbon bonds, using, for example, controlled porosity glass (CPG) as the layer material. Linker molecules also facilitate target recognition of the synthesized polymers. Furthermore, the linker molecules are preferably chosen based upon their hydrophilic/hydrophobic properties to improve presentation of synthesized polymers to certain receptors. For example, in the case of a hydrophilic receptor, hydrophilic linker molecules will be preferred so as to permit the receptor to approach more closely the synthesized polymer.

The linker molecules are preferably of sufficient length to permit polymers on a completed substrate to interact freely with binding entities exposed to the substrate. The linker molecules, when used, are preferably 10 to 1000 atoms long, and in especially preferred embodiments are about 650 atoms long to provide sufficient exposure of the functional groups to the binding entity. The linker molecules, which may be advantageously used in accordance with the invention include, for example, aryl acetylene, ethylene glycol oligomers containing from 2 to 10 monomer units, diamines, diacids, amino acids, and combinations thereof. Other linker molecules may be used in accordance with the different embodiments of the present invention and will be recognized by those skilled in the art in light of this disclosure.

The linker molecules are preferably of sufficient length to permit polymers such as peptides on a completed substrate to interact freely with binding entities exposed to the substrate. The linker molecules, when used, are most preferably about 650 atoms long to provide sufficient exposure of the functional groups to the binding entity. The linker molecules, which may be advantageously used in accordance with the invention include, for example, aryl acetylene, ethylene glycol oligomers containing from 2 to 10 monomer units, diamines, diacids, amino acids, and combinations thereof. Other linker molecules may be used in accordance with the different embodiments of the present invention and will be recognized by those skilled in the art in light of this disclosure.

According to another preferred embodiment, linker molecules may be provided with a cleavable group at an intermediate position, which group can be cleaved with an electrochemically generated reagent. This group is preferably cleaved with a reagent different from the reagent(s) used to remove the protective groups. This enables removal of the various

synthesized polymers or peptides following completion of the synthesis by way of electrochemically generated reagents. In particular, derivatives of the acid labile 4,4'-dimethyoxytrityl molecules with an exocyclic active ester can be used in accordance with the present invention. These linker molecules can be obtained from Perseptive Biosystems, Framingham, Massachusetts. More preferably, N-succinimidyl-4-[bis-(4-methoxyphenyl)-chloromethyl]-benzoate is used as a cleavable linker molecule during DNA synthesis. The synthesis and use of this molecule is described in A *Versatile Acid-Labile Linker for Modification of Synthetic Biomolecules,* by Brian D. Gildea, James M. Coull and Hubert Koester, Tetrahedron Letters, Volume 31, No. 49, pgs 7095-7098 (1990). Alternatively, other manners of cleaving can be used over the entire array at the same time, such as chemical reagents, light or heat.

The use of cleavable linker groups affords dissociation or separation of synthesized molecules, *e.g.,* polymers or amino acid sequences, from the electrode array at any desired time. This dissociation allows transfer of the, for example, synthesized polymer or amino acid sequence, to another electrode array or to a second substrate. Obviously, those skilled in the art can contemplate several uses for transferring the molecules synthesized on the original electrode to a second substrate.

The arrays according to the present invention need not be in any specific shape, that is, the electrodes need not be in a square matrix shape. Contemplated electrode array geometries include: squares; rectangles; rectilinear and hexagonal grid arrays with any sort of polygon boundary; concentric circle grid geometries wherein the electrodes form concentric circles about a common center, and which may be bounded by an arbitrary polygon; and fractal grid array geometries having electrodes with the same or different diameters. Interlaced electrodes may also be used in accordance with the present invention. Preferably, however, the array of electrodes contains at least 100 electrodes in an at least 10x10 matrix. More preferably, the array of electrodes contains at least 400 electrodes in, for example, an at least 20x20 matrix. Even more preferably, the array contains at least 1024 or 2048 electrodes in, for example, an at least 64x32 matrix, and still more preferably, the array contains at least 204,800 electrodes in, for example, an at least 640x320 array. Other sized arrays that may be used in accordance with the present invention will be readily apparent to those of skill in the art upon review of this disclosure.

Electrode arrays containing electrodes ranging in diameter from approximately less than 1 micron to approximately 100 microns (0.1 millimeters) are advantageously used in accordance with the present invention. Further, electrode arrays having a distance of approximately 10-1000 microns from center to center of the electrodes, regardless of the electrode diameter, are

advantageously used in accordance with the present invention. More preferably, a distance of 50-100 microns exists between the centers of two neighboring electrodes.

The electrodes may be flush with the surface of the substrate. However, in accordance with a preferred embodiment of the present invention, the electrodes are hemisphere shaped, rather than flat disks. More specifically, the profile of the hemisphere shaped electrodes is represented by an arctangent function that looks like a hemisphere. Those skilled in the art will be familiar with electrodes of this shape. Hemisphere shaped electrodes help assure that the electric potential is constant across the radial profile of the electrode. That is, hemisphere shaped electrodes help assure that the electric potential is not larger near the edge of the electrode than in the middle of the electrode, thus assuring that the generation of electrochemical reagents occurs at the same rate at all parts of the electrode.

Electrodes that may be used in accordance with the invention may be composed of, but are not limited to, noble metals such as iridium and/or platinum, and other metals, such as, palladium, gold, silver, copper, mercury, nickel, zinc, titanium, tungsten, aluminum, as well as alloys of various metals, and other conducting materials, such as, carbon, including glassy carbon, reticulated vitreous carbon, basal plane graphite, edge plane graphite and graphite. Doped oxides such as indium tin oxide, and semiconductors such as silicon oxide and gallium arsenide are also contemplated. Additionally, the electrodes may be composed of conducting polymers, metal doped polymers, conducting ceramics and conducting clays. Among the noble metals, platinum and palladium are especially preferred because of the advantageous properties associated with their ability to absorb hydrogen, *i.e.*, their ability to be "preloaded" with hydrogen before being used in the methods of the invention.

In accordance with other preferred embodiments of the present invention, one or more of the electrodes are proximate to a "getter" structure. Preferably the "getter" structure comprises a second electrode. The second electrode may be of any shape or size. However, it may function to scavenge electrochemically generated reagents alone or in conjunction with a scavenging solution and/or a buffering solution or it may function to reduce or eliminate diffusion of ions into nearby electric sources such as semiconductor circuitry. Such second electrodes may be made of the same material as the selected electrodes discussed above.

The electrode(s) used in accordance with the invention may be connected to an electric source in any known manner. Preferred ways of connecting the electrodes to the electric source include CMOS switching circuitry, radio and microwave frequency addressable switches, light addressable switches, and direct connection from an electrode to a bond pad on the perimeter of a semiconductor chip. The placement of a "getter" structure in accordance with the description set

forth above effectively prolongs the life of a semiconductor chip thereby making such a connection particulary advantageous.

CMOS switching circuitry involves the connection of each of the electrodes to a CMOS transistor switch. The switch is accessed by sending an electronic address signal down a common bus to SRAM (static random access memory) circuitry associated with each electrode. When the switch is "on", the electrode is connected to an electric source. This is a preferred mode of operation.

Radio and microwave frequency addressable switches involve the electrodes being switched by a RF or microwave signal. This allows the switches to be thrown both with and/or without using switching logic. The switches can be tuned to receive a particular frequency or modulation frequency and switch without switching logic. Alternatively, the switches can use both methods.

Light addressable switches are switched by light. In this method, the electrodes can also be switched with and without switching logic. The light signal can be spatially localized to afford switching without switching logic. This is accomplished, for example, by scanning a laser beam over the electrode array; the electrode being switched each time the laser illuminates it. Alternatively, the whole array can be flood illuminated and the light signal can be temporally modulated to generate a coded signal. However, switching logic is required for flood illumination.

One can also perform a type of light addressable switching in an indirect way. In this method, the electrodes are formed from semiconductor materials. The semiconductor electrodes are then biased below their threshold voltage. At sufficiently low biases, there is no electrochemistry occurring because the electrons do not have enough energy to overcome the band gap. The electrodes that are "on" will already have been switched on by another method. When the electrodes are illuminated, the electrons will acquire enough energy from the light to overcome the band gap and cause electrochemistry to occur.

Thus, an array of electrodes can be poised to perform electrochemistry whenever they are illuminated. With this method, the whole array can be flood illuminated or each electrode can be illuminated separately. This technique is useful for very rapid pulsing of the electrochemistry without the need for fast switching electronics. Direct connection from an electrode to a bond pad on the perimeter of the semiconductor chip is another possibility, although this method of connection could limit the density of the array.

Electrochemical generation of the desired type of chemical species requires that the electric potential of each electrode have a certain minimum value. That is to say, a certain

minimum potential is necessary, which may be achieved by specifying either the voltage or the current. Thus, there are two ways to achieve the necessary minimum potential at each electrode: either the voltage may be specified at the necessary value or the current can be determined such that it is sufficient to accommodate the necessary voltage. The necessary minimum potential value will be determined by the type of chemical reagent chosen to be generated. One skilled in the art can easily determine the necessary voltage and/or current to be used based on the chemical species desired. The maximum value of potential that can be used is also determined by the chemical species desired. If the maximum value of potential associated with the desired chemical species is exceeded, undesired chemical species may be resultantly produced.

In a second aspect, the present invention features a method for determining the presence of a gene product in a sample. The method features contacting a biological sample with a peptide array. Preferably, the peptide array comprises at least about peptide probes. Especially preferably, the peptide array comprises at least about 1000 peptide probes. The method is preferably performed under conditions suitable to allow binding of gene products to corresponding ligands or binding domains. Preferably, the detection is performed using fluorescently labeled tags that may be detected by such devices as epifluorescent microscopes and a CCD camera.

Gene products may be separated by their binding affinity to a spatially defined microarray of small peptide probes. **Figure 3** demonstrates the methods employed generally. First, an array of peptide probes is synthesized. The probe set is designed to be sufficiently large and diverse that proteins bind selectively to individual peptide probes. The peptide array is then contacted with samples of interest. To compare the protein expression levels in two samples, each sample is preferably prederivatized with a different fluorescent dye. The samples are mixed and contacted to the array. The bound proteins are visualized by fluorescence microscopy, and the image captured by a suitable device such as a CCD camera. Peptide affinity probe arrays may be used to gather functional information about gene products subsequent to identifying the presence of such gene product. The amino acid sequence of a protein that binds to a given peptide probe may be determined by procedures well known to those skilled in the art, such as by MALDI-TOF mass spectroscopy. The sequence information may be compared to the sequences of know proteins to determine if the protein is new. If the protein is found to be previously unknown, structural and functional information may be inferred by comparing the homology of its amino acid sequence to the amino acid sequences of known proteins ((Bork et al., *Curr. Opin. Biotech* 4:393-403 (1994); Holm et al., *Science* 273:595-602 (1996); Johnson et al., *Crit. Rev. Biochem. and Mol. Bio.* 29:1-68 (1994); Sali, *Curr. Opin. Biotech* 6:437-451 (1995)). Clues about how the bound protein

interacts with other proteins may be drawn from the structure of the peptide probe(s) to which it binds (Jones, *Curr. Opin. Biotech.* 6:452 (1995)). The peptide probe may also be used as a lead compound in the design of drugs that inhibit the action of the protein. The use of the peptide probe to gather functional information about the protein requires that the binding between the probe and the protein be specific. Binding specificity is determined by immobilizing affinity probes in a concentration gradient. Specific binding of a gene product to a probe gives a signal that is proportional to the probe concentration.

In other embodiments, electrochemical methods may be used to detect and quantitate the presence of gene products at each individually addressable electrode on the array. Such electrochemical methods may be used to detect the gene product directly or indirectly. By way of example, a gene product may be labeled with an electrochemically active enzyme moiety such as β-galactosidase, glucose oxidase, or peroxidase. The labeled gene product may be detected by exposing the array to a solution containing the substrate for the enzyme, such as glucose or hydrogen peroxide, and a redox mediator such as hexacyanoferrate ions, ruthenium tris-bipyridyl ions or methyl viologen. Such a redox mediator shuttles electrons from each electrode to enzyme labeled gene products that are immobilized proximate to the electrode. The enzyme labeled gene products may be detected at each electrode by monitoring a shift in the open circuit potential due to reaction of the redox mediator with the enzyme. Alternatively, each electrode may be used to establish a constant potential, and the current necessary to maintain that potential may be monitored. As will be evident to one skilled in the art, many other electrochemical techniques such as cyclic voltammetry, chronocoulometry, or staircase voltammetry may be used to detect and quantify gene products at each individually addressable electrode of the array.

The methods of the present invention featuring array based examination of gene products by affinity binding of a sample to an immobilized peptide probe provides several advantages over the prior art. First, gene products that are difficult to observe by two dimensional electrophoresis, such as hydrophobic, basic, and large proteins, can be observed. Second, the ability to conduct a two color assay on the chip provides an internal standard that makes it possible to reliably quantify protein expression levels. Variability resulting from differences in sample preparation and differences in the susceptibility of proteins to stain is reduced. A dilution -series of affinity probes also acts as internal standard and to identify cross reactivity. Third, in particular embodiments wherein peptide probes are placed on an array in a defined pattern, the need for complex pattern recognition software is eliminated. Fourth, a peptide array allows gene product expression levels, amino acid sequence and potential interactions between the gene product and other peptides to be determined.

The methods of the present invention significantly reduce the time required to develop new drugs. Pharmaceutical treatment of disease results from the ability to develop molecular methods that inhibit deleterious cellular functions. The discovery of new pharmaceutical treatments is facilitated by an ability to identify rapidly the molecular basis of a disease and to develop rapidly an inhibitory agent. Because of the complexity of biological systems, any method that simultaneously observes multiple parameters greatly facilitates the speed and effectiveness of the discovery process. The peptide probe arrays described herein provide the potential to both discover new drug targets and to identify lead compounds, in the form of the immobilized probe, which can serve as the basis for investigations by medicinal chemists.

In a third aspect, the present invention features a method for quantifying the amount of a gene product in a sample. The method features contacting a biological sample with a peptide array. Preferably, the peptide array comprises at least about 100 peptide probes. Especially preferably, the peptide array comprises at least about 1000 peptide probes. The method is preferably performed under conditions suitable to allow binding of gene products to corresponding ligands or binding domains.

The term "quantifying" as used herein includes absolute and relative quantification. Absolute quantification may be accomplished by inclusion of known concentration(s) of one or more target amino acid sequence. Alternatively, relative quantification can be accomplished by comparing hybridization signals between two or more target amino acids.

The present invention features a method to quantitate gene product expression by interrogating a microarray of peptide affinity probes with labeled gene products, preferably fluorescently labeled. The experimental sample and a control sample are labeled with two different labels such as fluorescent dyes and mixed together. The mixture may be used to interrogate an affinity array of peptide probes. Gene product expression may be quantitated by determining the ratio of the two label signals. The labels may be incorporated by any of a number of means well known to those of skill in the art.

A label may be added directly to the original gene product sample. Means of attaching labels to amino acids, peptides and proteins are well known to those of skill in the art. Detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical, laser or chemical means. Useful labels in the present invention include biotin for staining with labeled streptavidin conjugate, magnetic beads (*e.g.*, Dynabeads™), fluorescent dyes (*e.g.*, fluorescein, texas red, rhodamine, green fluorescent protein, etc.), radiolabels (*e.g.*, 3H, '251, 35S, 14C, 32P, etc.), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase, etc.), and colorimetric labels such as

colloidal gold or colored glass or plastic (*e.g.*, polystyrene, polypropylene, latex, etc.) beads. Uses of such labels are provided in, *e.g.*, U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

Means of detecting such labels are well known to those of skill in the art. Radiolabels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted light. Enzymatic labels may be detected by providing the enzyme with a substrate and detecting the reaction product produced by the action of the enzyme on the substrate. Colorimetric labels may be detected by simply visualizing the colored label.

The label may be added to the target gene products prior to, or after binding to the peptide probes. Direct labels are detectable labels that are directly attached to or incorporated into the target (sample) gene products prior to binding to the peptide probe array. Indirect labels may be joined to the hybrid duplex after hybridization. In some embodiments, the indirect label is attached to a binding moiety that has been attached to the target peptides. Thus, for example, the target gene product may be biotinylated before the hybridization. After hybridization, an aviden-conjugated fluorophore will bind the biotin bearing hybrid duplexes providing a label that is easily detected.

In some embodiments, the target gene products are not themselves labeled. Rather, the peptide probes are labeled or are attached directly or indirectly to a signal resposive moiety. Methods for detecting labeled target gene products bound to the probes of a high density microarray are known to those of skill in the art. A colorimetric label or a radioactive labeled probe may be used.

In some embodiments, the target gene products are labeled with a fluorescent label and the localization of the label on the array may be accomplished by fluorescent microscopy. The hybridized array may be excited with a light source at the excitation wavelength of the particular fluorescent label and the resulting fluorescence at the emission wavelength is detected. In a particularly preferred embodiment, the excitation light source is a laser appropriate for the excitation of the fluorescent label. A confocal microscope may be automated with a computer-controlled stage to automatically scan the peptide array. Similarly, the microscope may be equipped with a phototransducer attached to an automated data acquisition system to automatically record the fluorescence signal produced by hybridization to each peptide probe on the array. Such automated systems are described at length in U.S. Patent No: 5,143,854.

One of skill in the art will appreciate that methods for evaluating the binding results varies with the nature of the specific probe as well as the controls provided. In one embodiment,

simple quantification of the intensity for each probe is determined. This is accomplished by measuring probe signal strength at each location representing a different probe on the peptide array. Comparing the absolute intensities of an array bound to a gene product from a test sample with intensities produced by a control sample provides a measure of the relative amount of the gene product present.

Signals may vary in strength with affinity, the amount of label on the sample gene product and the amount of the particular gene product in the sample. Typically gene products present at very low levels may produce a very weak signal. At some low levels of concentration, the signal may become indistinguishable from background. In evaluating the data, a threshold intensity value may be selected below which a signal is not counted as being essentially indistinguishable from background. Where it is desirable to detect gene products expressed at lower levels, a lower threshold may be chosen. Conversely, where only high expression levels are to be evaluated a higher threshold level may be selected. In one embodiment, a threshold is about 10% above that of the average background signal. In addition, the provision of appropriate controls permits a more detailed analysis that controls for variations in conditions. Thus, for example, in a preferred embodiment, the peptide array is provided with normalization controls. Where the binding conditions are poor, the normalization controls will show a smaller signal, and where binding conditions are good, the normalization controls will provide a higher signal. Normalization of the signal derived from other probes in the array to the normalization controls thus provides a control for variations in binding conditions. Typically, normalization is accomplished by dividing the measured signal from the other probes in the array by the average signal produced by the normalization controls. Normalization may also include correction for variations due to sample preparation and amplification. Such normalization may be accomplished by dividing the measured signal by the average signal from the sample preparation/amplification control probes. The resulting values may be multiplied by a constant value to scale the results.

The concentration of a particular sequence can then be determined by measuring the signal intensity of each of the probes that bind specifically to the target nucleic acid and normalizing to the normalization controls.

The methods of the present invention are preferably performed using a computer. The computer runs a software program that includes computer code incorporating the invention for analyzing binding intensities measured from a substrate and monitoring and/or quantifying the expression of one or more target gene products.

In a fourth aspect, the present invention features a method for identifying the functional binding domain of a gene product in a sample. . The method features contacting a biological

sample with a peptide array. Preferably, the peptide array comprises at least about 100 peptide probes. Especially preferably, the peptide array comprises at least about 1000 peptide probes. The method is preferably performed under conditions suitable to allow binding of gene products to corresponding ligands or binding domains.

Small peptide molecules are useful model systems for exploring the relationship between structure and function in biology. For example, the twenty naturally occurring amino acids can be condensed into polymeric molecules. These polymeric molecules form a large variety of three-dimensional spatial and electronic structures. Each structure arises from a particular amino acid sequence and solvent condition. The number of possible hexapeptides of the twenty naturally occurring amino acids, for example, is 206, or 64 million different peptides. As shown by epitope studies, the small peptide molecules are useful in target-binding studies, and sequences as short as a few amino acids are recognized with high specificity by some antibodies.

The process of discovering ligands with desirable patterns of specificity for targets of biological importance is central to many contemporary approaches to drug discovery. These approaches, based on structure-activity relationships, involve rational design of ligands and large scale screening of families of potential ligands. Often, a combination of approaches is used. The ligands are often, but not exclusively, small peptide molecules.

In some embodiments, the present invention provides a device for analyzing uncharacterized gene products. A spatially coded array of peptides, e.g. antibodies, may be positioned on the array. The array may then be used to obtain peptide sequence information. Preferred embodiments of this aspect of the invention may be prepared by immobilizing a microarray of peptides to a biocompatible support with a cleavable linker. The peptides can be either short sequences having minimal secondary structure, such as 5 to 10 amino acids, or longer sequences having secondary structure, such as 20 to 50 amino acids. Longer peptides or proteins may also be used. The amino acid sequence of the peptide is known by its location in the array.

According to these preferred embodiments, the arrays of the present invention may then be treated with an antibody phage library. The antibodies displayed on the phage binds to its corresponding peptide epitope. The random mixture of phage antibodies may be spatially sorted according to its binding epitope. Not all of the multiple copies of the antibody on the surface of the phage bind to the immobilized peptides. Some remain available for binding to the biological sample of proteins that are to be studied. The proteins in the sample to be studies may be labeled and contacted with the chip. They bind to locations where antibodies present to epitopes on the protein. Those of skill in the art will appreciate that if a labeled protein binds to a specific location, then it contains the sequence that is the same as the peptide immobilized in that location.

Non-specific interactions between the protein sample and the phage can be eliminated by the use of a dilution series of peptides.

The phage can be removed in individual areas where binding occurs by cleaving the linker, and additional phage can be produced by infecting bacteria with the cleaved phage. The selectivity of the displayed antibody can be determined by observing where the isolated phage binds on a fresh peptide array.

In a fifth aspect, the present invention features a method for producing an array of peptide probes for analyzing gene products. . Preferably, the array of peptide probes is formed on a porous membrane proximate at least one electrode. Preferably, the electrode current or potential is controlled and operated by interface with a computer either proximate or remote from the electrode. In preferred embodiments, the array of peptide probes is formed on a porous or non-solid support having a plurality of electrodes proximate the porous membrane. In especially preferred embodiments, at least about 100 electrodes and peptides are present on the array. In even more preferred embodiments at least about 500 or at least about 1000 electrodes and peptides are present on the array.

The only practical method to prepare a microarray of the large number of peptide affinity probes required to study the proteome is *in-situ* probe synthesis by combinatorial methods. The preparation of peptide microarrays requires the ability to remove selectively an amino protecting group from an immobilized amino acid. Previous efforts in the field have relied on photolithography to remove photolabile amino protecting groups. The photodeprotection technique has numerous drawbacks that include the necessity to synthesize precursor amino acids with photolabile protecting groups, exposure of synthesized peptides to intense UV irradiation for long periods of time, and the necessity to fabricate new and expensive photo masks for each peptide array that is to be synthesized. In addition, peptides made by this method are prepared as monolayers immobilized on a glass surface, a chemical environment quite dissimilar from the biological environments in which proteins usually fold and are active.

Figure 1 demonstrates how an array according to the present invention may be used to synthesize a pattern of molecules. First, the array is coated with a biocompatible porous membrane that allows molecules to flow freely between the bulk solvent and the electrode. The array is then immersed in a solution containing an inactive precursor to the electrochemically generated (ECG) reagent of interest. For peptide synthesis, this would be an ECG-reagent to remove amino protecting groups. A computer then turns on the desired electrode pattern, and the precursor is electrochemically converted into the active species. The ECG-reagent, in turn, reacts

with molecules immobilized to the membrane overlying the electrode. By buffering the solution properly, diffusion of the ECG-reagent beyond the area of the active electrode is eliminated.

The arrays of the present invention are preferably similar to those described in United States Serial Nos. 09/003,075 and 09/214,348, the disclosures of which are herein incorporated by reference. Such arrays are designed to allow synthesizing chemical compounds such as peptides at well-defined and individually addressable locations. Such arrays may be manufactured at low cost by contract fabricators using existing semiconductor manufacturing facilities. **Figure 1** describes how such an array may be used to synthesize a pattern of molecules. First, the array may be coated with a biocompatible porous membrane that allows molecules to flow freely between a bulk solvent and an electrode. The array may then be immersed in a solution containing a precursor to an electrochemically-generated (ECG) reagent of interest. For peptide synthesis, this is preferably an ECG-reagent to remove amino protecting groups. A computer may then interface with the array to turn on the desired electrode pattern, and the precursor may be electrochemically converted into an active species. The electrochemically-generated (ECG) reagent, in turn, reacts with molecules immobilized to the membrane overlying the electrode.

A central feature of preferred arrays according to the present invention is the ability to confine the ECG reagents to a region immediately adjacent to a selected microelectrode. This is illustrated in **Figure 4**. Here, a fluorescein dye has been immobilized covalently at individually addressed microelectrode locations. The dye may be tightly confined to a checkerboard pattern and exhibits substantially no chemical crosstalk between active and inactive microelectrodes. This level of localization of ECG reagents may be achieved by exploiting the physical chemistry of the solution in which the microelectrode array is immersed. Such solutions usually contain buffers and scavengers that react with ECG reagents. However, the rate at which ECG reagents are produced can overwhelm the ability of the solution to react with them in the small local area immediately proximate to the microelectrode. As a result, chemistry that is mediated by ECG reagents occurs near selected microelectrodes, but there is no chemical crosstalk.

The method of the present invention preferably utilizes a method for electrochemical placement of a material at a specific location on a substrate as described in United States Patent Application Serial Nos. 09/003,075 and 09/214,348, the disclosures of which are herein incorporated by reference, and in international patent application numbers PCT/US97/11463 and PCT/US99/00599, comprising the steps of: providing a substrate having at its surface at least one electrode that is proximate to at least one molecule that is reactive with an electrochemically generated reagent, applying a potential to the electrode sufficient to generate electrochemical reagents capable of reacting to the at least one molecule proximate to the electrode, and producing

a chemical reaction thereby. Such method allows production of an array of peptide probes comprising individual amino acids.

In some preferred embodiments, the present invention utilizes a method for the electrochemical placement of a material at a specific location on a substrate comprising the steps of: providing a substrate having at its surface at least one electrode that is proximate to at least one molecule bearing at least one protected chemical functional group, applying a potential to the electrode sufficient to generate electrochemical reagents capable of deprotecting at least one of the protected chemical functional groups of the molecule, and bonding the deprotected chemical functional group with a monomer or a pre-formed molecule. According to the present invention, the monomer or molecule is normally an amino acid.

In other preferred embodiments, the present invention utilizes a method for electrochemical synthesis of an array of separately formed peptides on a substrate, which comprises the steps of: placing a buffering or scavenging solution in contact with an array of electrodes that is proximate to a substrate surface, said surface being proximate to one or more molecules bearing at least one protected chemical functional group attached thereto, selectively deprotecting at least one protected chemical functional group on at least one of the molecules; bonding a first monomer, normally an amino acid, having at least one protected chemical functional group to one or more deprotected chemical functional groups of the molecule; selectively deprotecting a chemical functional group on the bonded molecule or another of the molecules bearing at least one protected chemical functional group; bonding a second monomer, normally an amino acid, having at least one protected chemical functional group to a deprotected chemical functional group of the bonded molecule or the other deprotected molecule; and repeating the selective deprotection of a chemical functional group on a bonded protected monomer or a bonded protected molecule and the subsequent bonding of an additional monomer, normally an amino acid, to the deprotected chemical functional group until at least two separate polymers, normally peptides, of desired length are formed on the substrate surface. According to the present invention, the monomer or molecule is normally an amino acid and the polymer is a peptide.

In additional preferred embodiments, the present invention utilizes a method for electrochemical synthesis of an array of separately formed polymers on a substrate, which comprises the steps of: placing a buffering or scavenging solution in contact with an array of electrodes that is proximate to a substrate surface, said surface being proximate to one or more molecules bearing at least one protected chemical functional group attached thereto, selectively deprotecting at least one protected chemical functional group on at least one of the molecules;

bonding a first monomer having at least one protected chemical functional group to one or more deprotected chemical functional groups of the molecule; selectively deprotecting a chemical functional group on the bonded molecule or another of the molecules bearing at least one protected chemical functional group; bonding a second monomer having at least one protected chemical functional group to a deprotected chemical functional group of the bonded molecule or the other deprotected molecule; and repeating the selective deprotection of a chemical functional group on a bonded protected monomer or a bonded protected molecule and the subsequent bonding of an additional monomer to the deprotected chemical functional group until at least two separate polymers of desired length are formed on the substrate surface. According to the present invention, the monomer or molecule is normally an amino acid and the polymer is a peptide.

In further preferred embodiments, the methods of the present invention utilize a "getter" structure such as a second electrode proximate to the array of electrodes or proximate to each of the electrodes individually. Such a "getter" structure may reduce chemical crosstalk between adjacent electrodes and/or prolong the life of semiconductor circuitry. Various semiconductor circuitry may be placed in a manner to control electrodes individually or corporately according to any one of the methods that are well known in the art. A "getter" structure in accordance with the present invention may be placed in an appropriate location either exposed to the external environment or internal to a semiconducting device.

By using the electrochemical techniques discussed herein, it is possible to place monomers, both those that can be used for polymer synthesis and those that can be decorated, and pre-formed molecules at small and precisely known locations on a substrate. It is therefore possible to synthesize peptides of a known amino acid sequence at selected locations on a substrate. For example, in accordance with the presently disclosed invention, one can place amino acids at selected locations on a substrate to synthesize desired sequences of amino acids in the form of peptides.

In order to form the peptide arrays of the present invention, monomer amino acids with or without linker molecules may be placed at a specific location on a substrate. The present invention features synthesizing amino acid polymers at a specific location on a substrate, and in particular polypeptides, by means of a solid phase polymerization technique, which generally involves the electrochemical removal of a protecting group from a molecule provided on a substrate that is proximate at least one electrode.

Electrochemical reagents capable of electrochemically removing protecting groups from chemical functional groups on the molecule are generated at selected electrodes by applying a sufficient electrical potential to the selected electrodes. Removal of a protecting group, or

"deprotection," in accordance with the invention, occurs at selected molecules when a chemical reagent generated by the electrode acts to deprotect or remove, for example, an acid or base labile protecting group from the selected molecules.

In one embodiment of the present invention, a terminal end of a monomer amino acid, or linker molecule (*i.e.*, a molecule which "links," for example, a monomer to a substrate) is provided with at least one reactive functional group, which is protected with a protecting group removable by an electrochemically generated reagent. The protecting group(s) is exposed to reagents electrochemically generated at the electrode and removed from the monomer, amino acid or linker molecule in a first selected region to expose a reactive functional group. The substrate is then contacted with a first monomer or pre-formed molecule, which bonds with the exposed functional group(s). This first monomer or pre-formed molecule may also bear at least one protected chemical functional group removable by an electrochemically generated reagent.

The monomers or pre-formed molecules, normally amino acids, can then be deprotected in the same manner to yield a second set of reactive chemical functional groups. A second monomer or pre-formed molecule, normally an amino acid, which may also bear at least one protecting group removable by an electrochemically generated reagent, is subsequently brought into contact with the substrate to bond with the second set of exposed functional groups. Any unreacted functional groups can optionally be capped at any point during the synthesis process. The deprotection and bonding steps can be repeated sequentially at this site on the substrate until peptides or polymers of a desired sequence and length are obtained.

In another embodiment of the present invention, the substrate having one or more molecules bearing at least one protected chemical functional group bonded thereto is proximate an array of electrodes, which array is in contact with a buffering or scavenging solution. Following application of an electric potential to selected electrodes in the array sufficient to generate electrochemical reagents capable of deprotecting the protected chemical functional groups, molecules proximate the selected electrodes are deprotected to expose reactive functional groups, thereby preparing them for bonding. A monomer solution or a solution of pre-formed molecules, such as amino acids, peptides, proteins, etc. is then contacted with the substrate surface and the monomers or pre-formed molecules bond with the deprotected chemical functional groups.

Another sufficient potential is subsequently applied to select electrodes in the array to deprotect at least one chemical functional group on the bonded molecule or another of the molecules bearing at least one protected chemical functional group. A second monomer or pre-formed molecule, normally an amino acid, having at least one protected chemical functional group is subsequently bonded to a deprotected chemical functional group of the bonded molecule

or the other deprotected molecule. The selective deprotection and bonding steps can be repeated sequentially until peptides of a desired sequence and length are obtained. The selective deprotection step is repeated by applying another potential sufficient to effect deprotection of a chemical functional group on a bonded protected monomer or a bonded protected molecule. The subsequent bonding of an additional monomer or pre-formed molecule, normally an amino acid, to the deprotected chemical functional group(s) until at least two separate polymers of desired length are formed on the substrate. Preferred embodiments of the methods of the present invention use a buffering or scavenging solution in contact with each electrode, which is buffered towards the electrochemically generated reagents, in particular, towards protons and/or hydroxyl ions, and that actively prevents chemical cross-talk caused by diffusion of the electrochemically generated ions from one electrode to another electrode in an array. For example, when an electrode exposed to an aqueous or partially aqueous media is biased to a sufficiently positive (or negative) potential, protons (or hydroxyl ions) are produced as products of water hydrolysis. Protons, for example, are useful for removing electrochemical protecting groups from several molecules useful in combinatorial synthesis, for example, peptides.

In order to produce separate and pure peptides, it is desirable to keep these protons (or hydroxyl ions) confined to the area immediately proximate the selected electrode(s) in order to minimize, and, if possible to eliminate, chemical cross-talk between nearby electrodes in an array. The spatial extent of excursion of electrochemically generated reagents can be actively controlled by the use of a buffering or scavenging solution that reacts with the reagents that move away from the selected electrodes, thus preventing these reagents from reacting at a nearby electrode.

Another technique for confining these electrochemically generated reagents to the area immediately proximate the selected electrode(s) is to place a "getter" structure in proximity to the selected electrode(s) and substantially exposed to the external environment. Such a "getter" structure may be used in conjunction with or in place of a scavenging solution. A "getter" structure may be designed of any suitable material and formed into any suitable shape or size as skilled artisans will readily appreciate. The most important criteria for such a "getter" structure is that it function to scavenge electrochemically generated reagents that may diffuse away from the selected electrode(s). The "getter" structure may function passively by reacting chemically with the electrochemically generated reagents. Alternatively, the "getter" structure may function actively to scavenge the electrochemically generated reagents. This may be performed by applying sufficient potential to the "getter" structure to cause electrochemical scavenging. Another function of the "getter" structure may be to prevent the diffusion of ions toward or into circuitry such as transistors that may be operably linked to the selected electrode(s). In

accordance with this function, the "getter" structure may be placed substantially at the interface between an insulating dielectric and a metallization layer operably linked to the selected electrode(s).

The substrate in the invention is proximate to at least one electrode, *i.e.*, an electrically conducting region of the substrate that is substantially surrounded by an electrically insulating region. The electrode(s), by being "proximate" to the substrate, can be located at the substrate, *i.e.*, embedded in or on the substrate, can be next to, below, or above the substrate, but need to be in close enough proximity to the substrate so that the reagents electrochemically generated at the electrode(s) can accomplish the desired deprotection of the chemical functional groups on the monomer(s) and/or molecule(s).

In addition to being proximate to at least one electrode, the substrate has on a surface thereof, at least one molecule, and preferably several molecules, bearing at least one chemical functional group protected by an electrochemically removable protecting group. These molecules bearing protected chemical functional groups also need to be proximate to the electrode(s). In this regard, the molecules on the surface of the substrate need to be in close enough proximity to the electrode(s) so that the electrochemical reagents generated at the electrode can remove the protecting group from at least one protected functional group on the proximate molecule(s).

The molecules bearing a protected chemical functional group that are attached to the surface of the substrate may be selected generally from monomers, linker molecules and pre-formed molecules. Preferably, the molecules attached to the surface of the substrate include monomers, amino acids, peptides, and linker molecules. All of these molecules generally bond to the substrate by covalent bonds or ionic interactions. Alternatively, all of these molecules can be bonded, also by covalent bonds or ionic interactions, to a layer overlaying the substrate, for example, a permeable membrane layer, which layer can be adhered to the substrate surface in several different ways, including covalent bonding, ionic interactions, dispersive interactions and hydrophilic or hydrophobic interactions. In still another manner of attachment, a monomer or pre- formed molecule may be bonded to a linker molecule that is bonded to either the substrate or a layer overlaying the substrate.

The monomers, linker molecules and pre-formed molecules used herein, preferably amino acids, are preferably provided with a chemical functional group that is protected by a protecting group removable by electrochemically generated reagents. If a chemical functional group capable of being deprotected by an electrochemically generated reagent is not present on the molecule on the substrate surface, bonding of subsequent monomers or pre-formed molecules cannot occur at this molecule. Preferably, the protecting group is on the distal or terminal end of the linker

molecule, monomer, or pre-formed molecule, opposite the substrate. That is, the linker molecule preferably terminates in a chemical functional group, such as an amino or carboxy acid group, bearing an electrochemically removable protective group. Chemical functional groups that are found on the monomers, linker molecules and pre-formed molecules include any chemically reactive functionality. Usually, chemical functional groups are associated with corresponding protective groups and will be chosen or utilized based on the product being synthesized. The molecules of the invention bond to deprotected chemical functional groups by covalent bonds or ionic interactions.

Monomers, particularly amino acids, used in accordance with the methods of the present invention to synthesize the various polymers, particularly peptides, contemplated include all members of the set of small molecules that can be joined together to form a polymer. This set includes, but is not limited to, the set of common L-amino acids, the set of D-amino acids and the set of synthetic amino acids. Monomers include any member of a basis set for synthesis of a polymer. For example, trimers of L-amino acids form a basis set of approximately 8000 monomers for synthesis of polypeptides. Different basis sets of monomers may be used at successive steps in the synthesis of a polymer. The number of monomers that can be used in accordance with the synthesis methods can vary widely, for example from 2 to several thousand monomers can be used, but in more preferred embodiments, the number of monomers will range from approximately 4 to approximately 200, and, more preferably, the number of monomers will range from 4-20.

In a preferred embodiment of the invention, the monomers are amino acids containing a protective group at its amino or carboxy terminus that is removable by an electrochemically generated reagent. A polymer in which the monomers are alpha amino acids and are joined together through amide bonds is a peptide, also known as a polypeptide. In the context of the present invention, it should be appreciated that the amino acids may be the L-optical isomer or the D-optical isomer or a mixture of the two. Peptides are at least two amino acid monomers long, and often are more than 20 amino acid monomers long.

Furthermore, essentially any pre-formed molecule can be bonded to the substrate, a layer overlaying the substrate, a monomer or a linker molecule. Pre- formed molecules include, for example, proteins, including in particular, receptors, enzymes, ion channels, and antibodies, nucleic acids, polysaccharides, porphyrins, and the like. Pre-formed molecules are, in general, formed at a site other than on the substrate of the invention. In a preferred embodiment, a pre-formed molecule is bonded to a deprotected functional group on a molecule, monomer, or another pre- formed molecule. In this regard, a pre-formed molecule that is already attached to

the substrate may additionally bear at least one protected chemical functional group to which a monomer or other pre-formed molecule may bond, following deprotection of the chemical functional group.

Protective groups are materials that bind to a monomer, a linker molecule or a pre-formed molecule to protect a reactive functionality on the monomer, linker molecule or pre-formed molecule, which may be removed upon selective exposure to an activator, such as an electrochemically generated reagent. Protective groups that may be used in accordance with the present invention preferably include all acid and base labile protecting groups. For example, peptide amine groups are preferably protected by t-butyloxycarbonyl (BOC) or benzyloxycarbonyl (CBZ), both of which are acid labile, or by 9-fluorenylmethoxycarbonyl (FMOC), which is base labile. Additionally, hydroxy groups on phosphoramidites may be protected by dimethoxytrityl (DMT), which is acid labile. Exocyclic amine groups on nucleosides, in particular on phosphoramidites, are preferably protected by dimethylformamidine on the adenosine and guanosine bases, and isobutyryl on the cytidine bases, both of which are base labile protecting groups. This protection strategy is known as fast oligonucleotide deprotection (FOD). Phosphoramidites protected in this manner are known as FOD phosphoramidites.

Additional protecting groups that may be used in accordance with the present invention include acid labile groups for protecting amino moieties: tert-butyloxycarbonyl, tert-amyloxycarbonyl, adamantyloxycarbonyl, 1-methylcyclobutyloxycarbonyl, 2-(p-biphenyl)propyl(2)oxycarbonyl, 2- (p-phenylazophenylyl)propyl(2)oxycarbonyl, α,α-dimethyl- 3,5-dimethyloxybenzyloxy-carbonyl, 2-phenylpropyl(2)oxycarbonyl, 4-methyloxybenzyloxycarbonyl, benzyloxycarbonyl, furfuryloxycarbonyl, triphenylmethyl (trityl), p-toluenesulfenylaminocarbonyl, dimethylphosphinothioyl, diphenylphosphinothioyl, 2-benzoyl-1-methylvinyl, o-nitrophenylsulfenyl, and 1- naphthylidene; as base labile groups for protecting amino moieties: 9- fluorenylmethyloxycarbonyl, methylsulfonylethyloxycarbonyl, and 5- benzisoazolylmethyleneoxycarbonyl; as groups for protecting amino moieties that are labile when reduced: dithiasuccinoyl, p-toluene sulfonyl, and piperidino-oxycarbonyl; as groups for protecting amino moieties that are labile when oxidized: (ethylthio)carbonyl; as groups for protecting amino moieties that are labile to miscellaneous reagents, the appropriate agent is listed in parenthesis after the group: phthaloyl (hydrazine), trifluoroacetyl (piperidine), and chloroacetyl (2- aminothiophenol); acid labile groups for protecting carboxylic acids: tert-butyl ester; acid labile groups for protecting hydroxyl groups: dimethyltrityl; and basic labile groups for protecting phosphotriester groups: cyanoethyl.

As mentioned above, any unreacted deprotected chemical functional groups may be capped at any point during a synthesis reaction to avoid or to prevent further bonding at such molecule. Capping groups "cap" deprotected functional groups by, for example, binding with the unreacted amino functions to form amides. Capping agents suitable for use in the present invention include: acetic anhydride, n-acetylimidizole, isopropenyl formate, fluorescamine , 3-nitrophthalic anhydride and 3-sulfoproponic anhydride. Of these, acetic anhydride and n-acetylimidizole are preferred.

The molecules of the invention, *i.e.,* the monomers, linker molecules and pre- formed molecules, can be attached directly to the substrate or can be attached to a layer or membrane of separating material that overlays the substrate. Materials that can form a layer or membrane overlaying the substrate, such that molecules can be bound there for modification by electrochemically generated reagents, include: controlled porosity glass (CPG); generic polymers, such as, teflons, nylons, polycarbonates, polystyrenes, polyacylates, polycyanoacrylates, polyvinyl alcohols, polyamides, polyimides, polysiloxanes, polysilicones, polynitriles, polyelectrolytes, hydrogels, epoxy polymers' melamines, urethanes and copolymers and mixtures of these and other polymers; biologically derived polymers, such as, polysaccharides, polyhyaluric acids, celluloses, and chitons; ceramics, such as, alumina, metal oxides, clays, and zeolites; surfactants; thiols; self-assembled monolayers; porous carbon; and fullerine materials. The membrane can be coated onto the substrate by spin coating, dip coating or manual application, or any other art acceptable form of coating.

Reagents that can be generated electrochemically at the electrodes fall into two broad classes: oxidants and reductants. There are also miscellaneous reagents that are useful in accordance with the invention. Oxidants that can be generated electrochemically include iodine, iodate, periodic acid, hydrogen peroxide, hypochlorite, metavanadate, bromate, dichromate, cerium (IV), and permanganate. Reductants that can be generated electrochemicaily include chromium (II), ferrocyanide, thiols, thiosulfate, titanium (III), arsenic (III) and iron (II). The miscellaneous reagents include bromine, chloride, protons and hydroxyl ions. Among the foregoing reagents, protons, hydroxyl ions, iodine, bromine, chlorine and the thiols are preferred.

In accordance with preferred embodiments of the synthesis methods of the present invention, a buffering and/or scavenging solution is in contact with each electrode. The buffering and/or scavenging solutions that may be used in accordance with the invention are preferably buffered toward, or scavenge, ions such as protons and/or hydroxyl ions, although other electrochemically generated reagents capable of being buffered and/or scavenged are clearly contemplated. The buffering solution functions to prevent chemical cross- talk due to diffusion of

electrochemically generated reagents from one electrode in an array to another electrode in the array, while a scavenging solution functions to seek out and neutralize/deactivate the electrochemically generated reagents by binding or reacting with them. Thus, the spatial extent of excursion of electrochemically generated reagents can be actively controlled by the use of a buffering solution and/or a scavenging solution. In accordance with the invention, the buffering and scavenging solutions may be used independently or together. Preferably, a buffering solution is used because the capacity of a buffering solution is more easily maintained, as compared with a scavenging solution.

Buffering solutions that can be used in accordance with the present invention include all electrolyte salts used in aqueous or partially aqueous preparations. Buffering solutions preferably used in accordance with the present invention include: acetate buffers, which typically buffer around pH 5; borate buffers, which typically buffer around pH 8; carbonate buffers, which typically buffer around pH 9; citrate buffers, which typically buffer around pH 6; glycine buffers, which typically buffer around pH 3; HEPES buffers, which typically buffer around pH 7; MOPS buffers, which typically buffer around pH 7; phosphate buffers, which typically buffer around pH 7; TRIS buffers, which typically buffer around pH 8; and 0.1 M KI in solution, which buffers the iodine concentration by the equilibrium reaction $I_2 + I^- = I_3^-$, the equilibrium coefficient for this reaction being around 10^{-2}.

Alternatively, or in combination with a buffering solution, a scavenging solution may be used that contains species such as ternary amines that function as proton scavengers or sulfonic acids that function as hydroxyl ion scavengers in nonaqueous media. The rate at which a reagent/species is scavenged depends both on the intrinsic rate of the reaction occurring and on the concentration of the scavenger. For example, solvents make good scavengers because they are frequently present in high concentrations. Most molecules scavenge in a nonselective way, however, some molecules, such as superoxide dismutase and horseradish peroxidase, scavenge in a selective manner.

Of particular interest to the present invention are scavenger molecules that can scavenge the different reactive species commonly generated, for example, by water hydrolysis at electrodes, including hydroxyl radicals, superoxides, oxygen radicals, and hydrogen peroxide. Hydroxyl radicals are among the most reactive molecules known, their rate of reaction is diffusion controlled, that is, they react with the first reactant/species they encounter. When hydroxyl radicals are generated by water hydrolysis, the first molecule they usually encounter is a water molecule. For this reason, water is a rapid and effective scavenger of hydroxyl radicals. Superoxides are also a relatively reactive species, but can be stable in some nonaqueous or

partially aqueous solvents. In aqueous media, superoxides rapidly react with most molecules, including water. In many solvents, they can be scavenged selectively with superoxidase dismutase.

Oxygen radicals are a family of oxygen species that exist as free radicals. They can be scavenged by a wide variety of molecules such as water or ascorbic acid. Hydrogen peroxide is a relatively mild reactive species that is useful, in particular, in combinatorial synthesis. Hydrogen peroxide is scavenged by water and many types of oxidizing and reducing agents. The rate at which hydrogen peroxide is scavenged depends on the redox potential of the scavenger molecules being used. Hydrogen peroxide can also be scavenged selectively by horseradish peroxidase. Another electrochemically generated species that can be scavenged is iodine. Iodine is a mild oxidizing reagent that is also useful for combinatorial synthesis. Iodine can be scavenged by reaction with hydroxyl ions to form iodide ions and hypoiodite. The rate at which iodine is scavenged is pH dependent; higher pH solutions scavenge iodine faster. All of the scavenger molecules discussed above may be used in accordance with the present invention. Other scavenger molecules will be readily apparent to those skilled in the art upon review of this disclosure.

In accordance with the synthesis methods of the present invention, the buffering solutions are preferably used in a concentration of at least 0.01 mM. More preferably, the buffering solution is present in a concentration ranging from 1 to 100mM, and still more preferably, the buffering solution is present in a concentration ranging from 10 to 100mM. Most preferably, the buffering solution concentration is approximately 30 mM. A buffering solution concentration of approximately 0.1 molar, will allow protons or hydroxyl ions to move approximately 100 angstroms before buffering the pH to the bulk values. Lower buffering solution concentrations, such as 0.00001 molar, will allow ion excursion of approximately several microns, which still may be acceptable distance depending on the distance between electrodes in an array.

In accordance with the methods of the present invention, the concentration of scavenger molecules in a solution will depend on the specific scavenger molecules used since different scavenging molecules react at different rates. The more reactive the scavenger, the lower the concentration of scavenging solution needed, and vice versa. Those skilled in the art will be able to determine the appropriate concentration of scavenging solution depending upon the specific scavenger selected.

The at least one electrode proximate the substrate of the invention is preferably an array of electrodes. Arrays of electrodes of any dimension may be used, including arrays containing up to several million electrodes. Preferably, multiple electrodes in an array are simultaneously

addressable and controllable by an electrical source. More preferably, each electrode is individually addressable and controllable by its own electrical source, thereby affording selective application of different potentials to select electrodes in the array. In this regard, the electrodes can be described as "switchable".

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following are provided purely by way of example and are not intended to limit the scope of the present invention.

EXAMPLE 1

Materials and Methods

1. **Semiconductor Chip.** A porous microarray with 1024 spatially addressable electrodes.
2. **Application of Porous Membrane.** A hot (95 °C), solution of agarose in H_2O. (25 mg/mL) was applied to the array by spin coating at 2000 RPM's for one minute.
3. **Electrochemical Apparatus.** Interface designed to connect the array to a personal computer. Software allows for varying potentials, patterns, and reaction times times.
4. **Electrochemical Stock Solution.** A solution of vitamin B_{12} (2.8 mg/mL) and tetramethylamonium nitrate (TBAN, 13.3 mg/mL).
5. **Rinsing Protocol.** After electrochemically promoted reactions, the array was rinsed for 5-10 seconds with the solvent in the following order: ethanol, water, ethanol.
6. **Pulsing.** A method of applying intermittent voltage to optimize the electrochemical reaction. Pulsing described in the following experiments consists of 0.5 sec of applied potential followed by 0.1 sec of no potential. When listed, the duration of electrochemistry refers to the total amount of time the potential was applied.
7. **Staining.** Two separate amine labeling protocols were employed. Chips were soaked in either of the following solutions for 5 minutes. Fluorescein stain solution: fluorescein-NHS (100 μL, 3.5 mg/mL in DMF) and phosphate buffer (700 μL, pH 7, 10mM). Anthracene dialdehyde (ADA) solution: was prepared sodium borate buffer (450 μL, 10 mM), ADA (200 μL 1 mM) and KCN (200 μL, 0.1 M).

I. Synthesis

General. Preparing a molecular array on an electrochemical chip begins by coating the chip with a porous reaction layer in which the molecules of interest are synthesized. The chip is then immersed in a reaction solution that is inert until it is electrochemically activated. Electrodes are turned on under the areas of the solid support where chemistry is to occur. The electrode activates

the solution (either by oxidation or reduction) and produces a reagent that diffuses into the reaction layer. By formulating the solution with an appropriate buffer, diffusion of the reagent beyond the area immediately over the electrode is eliminated. Thus, the reagent is contained in a "virtual flask" without physical walls. After the required reaction time has elapsed, the electrodes are turned off, leaving behind the chemically modified solid support. See **Figure 5.**

Peptide synthesis on an electrode array begins by chemically attaching a linker molecule to the solid support over the electrodes. The linker molecule introduces a protected amino group onto the solid support from which the peptide will be synthesized, and allows for adjusting the distance between the peptide and the solid support. Distance may significantly affect the ability of the peptide to interact with biological macromolecules. The linker molecule can be either the first amino acid of the peptide, or a molecule designed to have a more stable linkage. **Figure 6** depicts the structure of an especially preferred Fmoc linker.

Site Selective synthesis of peptides on an electrode array can be performed by one of two general methods. In the first, an electrode array may be used to produce a reagent at the desired location that will remove a protecting group from an amine. Once the amine is deprotected, standard peptide bond forming chemistries are used to couple a desired amino acid to the activated amine. In the second method, the electrode array is used to produce a reagent, at the desired location, that enhances the rate of amide bond formation between an activated protected amino acid in solution and an amino group immobilized to solid support over the electrode. This results in the synthesis of a peptide with a protected amino group. The protecting group is removed by standard methods.

The following peptides were synthesized by standard methods: Fmoc-YGGFL-biotin, Fmoc-YGGFL-OH, and Fmoc-GGFL-OH. A Fmoc-carbonate linker was prepared.

Electrochemical Immobilization of Fmoc-Protected Glycine-OPfp. A chip was immersed in a solution containing 10 mg Fmoc-Gly-OPfp (dissolved in 200 μL DMF) and 800 μL electrochemical stock solution for immobilization. A potential of 2.0 V was applied for 5 min with standard pulsing. The array was rinsed by the standard method.

Electrochemical Immobilization of other Amino Acids and Peptides. The desired amino acid or peptide was dissolved in DMF (0.5 mL) and activated by adding diisopropylethyl amine ($(iPr)_2NEt$, 2, eq.) and. HBTU (1 eq). The solution was stirred for 5 minutes and N-hydroxysuccinimide (NHS-OH, 2 eq) was added. After stirring for 25 minutes the activated solution (200 μL) was added to electrochemical stock solution (800 μL). The chip was

immediately immersed in the solution and a potential of 2.4 V applied in a checkerboard pattern for 5 min, using standard pulsing.

Site Selective Removal of Fmoc-Groups with a Piperidine Salt. Site specific deprotection of Fmoc-groups can be performed by immersing the chip in a solution of a piperidine salt, for example piperidine hydrochloride. Cathodic reduction of the reagent produces base over the electrode that neutralizes the salt to give free piperidine which in turn removes the Fmoc-group. Standard solid-phase peptide synthesis techniques can then be employed to produce an amide bond with the free amine.

Site Selective Removal of Boc-Groups with Acid. Site specific deprotection of Boc-groups can be performed by immersing the chip in an aqueous solution. Anodic oxidation of water produces acid over the electrode, which in turn removes the Boc-group. Standard solid-phase peptide synthesis techniques can then be employed to produce an amide bond with the free amine.

Site Selective Removal of Fmoc-Groups with Azobenzene. Site specific deprotection of Fmoc-groups was performed by immersing the chip in a solution azobenzene. Cathodic reduction of the reagent produces base over the electrode. **Figure 7** shows how azobeneze enhances the deprotection of Fmoc-groups when compared to solvent, especially at lower voltages. Standard solid-phase peptide synthesis techniques were then employed to produce the amide bond. See **Figure 8.**

Site Selective Formation of Amide Bonds. The chip was immersed in a solution of an activated Fmoc-protected amino acid and electrolyte. The rate of amide bond formation is enhanced by turning on the electrode. A Fmoc-protected amine was immobilized in a checkerboard pattern over the top four rows of the chip. After the Fmoc-groups were removed by treating the chip with piperidine, amide bond formation between the resulting amines and Fmoc-protected glycine was electrochemically induced over the right hand column of three electrodes. The chip was then treated with an amino reactive dye (anthracene dialdehyde) and imaged under an epifluorecent microscope. The dark upper right hand corner indicates that reactive amino groups are no longer present. Amide bond formation is followed by standard Fmoc-deprotection with piperidine. See **Figure 9**.

Chip Bound Synthesis of Leu-Enkephalin. Fmoc-GGFL-OH was activated and immobilized on the array in stripes (as described above). Global deprotection of Fmoc groups was attained by

soaking in 20% piperdine in acetonitrile for 5 min. An orthogonal, overlapping immobilization pattern of Fmoc-Tyr-OH was run similarly. This reaction completed the five amino acid peptide leucine enkephalin, YGGFL. The final Fmoc-protecting groups were removed with piperidine. See **Figure 10**.

Antibody Assay

Recognition of Leu-Enkephalin. The array was immersed in a buffer solution containing monoclonal mouse anti-leu-enkephalin (clone 3-E7, Boeringer Mannheim), followed by Texas-Red labeled goat anti-mouse (Jackson ImmunoResearch). The secondary antibody was imaged with an epifluorescent microscope. Antibody binding to the desired peptide was ~3 fold greater than binding to tyrosine alone, and ~5 fold greater than binding to the tetrapeptide GGFL. See **Figure 11.**

Dilution series to adjust for non-specific interations. Non-specific interactions between immobilized affinity probes and biological samples complicate the interpretation of binding data. Specific and non-specific interaction can be distinguished by examination of the interaction between an affinity probe and a biological sample over a range of probe concentrations.

By varying the time an electrode is turned on, the array can be used to prepare a dilution series of an immobilized species. A dilution series of a Fmoc protected amino linker, on top of which peptides can be synthesized, was prepared by immobilization of an Fmoc protected carbonate linker on the array at a potential of 2.0 V for one to six minutes, in one minute intervals. After immobilization of the linker, the Fmoc-group was removed by treatment with piperidine, the resulting amino groups were fluorescently stained, and the chip was imaged under an epifluorescent microscope. The intensity of each spot was measured and plotted verses the amount of time the electrode was turned on. See **Figure 12**.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

WHAT IS CLAIMED IS:

1. An array of peptide probes comprising a porous membrane proximate at least one electrode.

2. An array of peptide probes according to claim 1 wherein the electrode current or potential is controlled and operated by interface with a computer.

3. An array of peptide probes according to claim 1 comprising at least about 100 electrodes.

4. An array of peptide probes according to claim 1 comprising at least about 1000 electrodes.

5. A method for determining the presence of a gene product in a sample comprising the steps of:

(a) preparing a peptide array on a porous substrate;

(b) contacting a biological sample with the peptide array;

(c) observing binding of the gene product to the peptide probe array.

6. The method of claim 5 wherein the array of peptide probes comprises at least about 100 peptides.

7. The method of claim 5 wherein the array of peptide probes comprises at least about 1000 peptides.

8. The method of claim 5 further comprising the step of labelling the gene product with a detectable label.

9. The method of claim 5 further comprising the step of labelling the gene product with a fluorescent label.

10. A method for quantifying the amount of a gene product in a sample comprising the steps of:

(a) preparing a peptide array on a porous substrate;

(d) contacting a biological sample with the peptide array;

(e) observing binding of the gene product to the peptide probe array.

11. The method of claim 10 wherein the array of peptide probes comprises at least about 100 peptides.

12. The method of claim 10 wherein the array of peptide probes comprises at least about 1000 peptides.

13. The method of claim 10 further comprising the step of labelling the gene product with a detectable label.

14. The method of claim 10 further comprising the step of labelling the gene product with a fluorescent label.

15. A method for identifying the functional binding domain of a gene product in a sample comprising the steps of:

(a) providing a spatially coded array of peptides;

(b) binding a plurality of antibodies to the peptides wherein the antibodies comprise a known amino acid sequence;

(c) contacting the array with a biological sample; and

(d) monitoring binding of molecules in the biological sample to the antibodies.

16. A method for producing an array of peptide probes for analyzing gene products comprising the steps of:.

(a) providing a substrate having at its surface at least one electrode that is proximate to at least one first amino acid bearing at least one protected chemical functional group,

(b) applying a potential to the electrode sufficient to generate electrochemical reagents capable of deprotecting at least one of the protected chemical functional groups of the first amino acid, and

(c) bonding the deprotected chemical functional group with a second amino acid.

17. A method for making an array of one or more peptide probes for analyzing gene products comprising the steps of:

(a) placing a buffering or scavenging solution in contact with an array of electrodes that is proximate to a substrate surface, said surface being proximate to one or more first amino acids bearing at least one protected chemical functional group attached thereto,

(b) selectively deprotecting at least one protected chemical functional group on at least one of the amino acids;

(c) bonding a second amino acid having at least one protected chemical functional group to one or more deprotected chemical functional groups of the first amino acid;

(d) repeating the selective deprotection of a chemical functional group on a bonded protected amino acid until at least two separate peptides of desired length are formed on the substrate surface.

18. A method for making an array of peptide probes for analyzing gene products comprising the steps of:

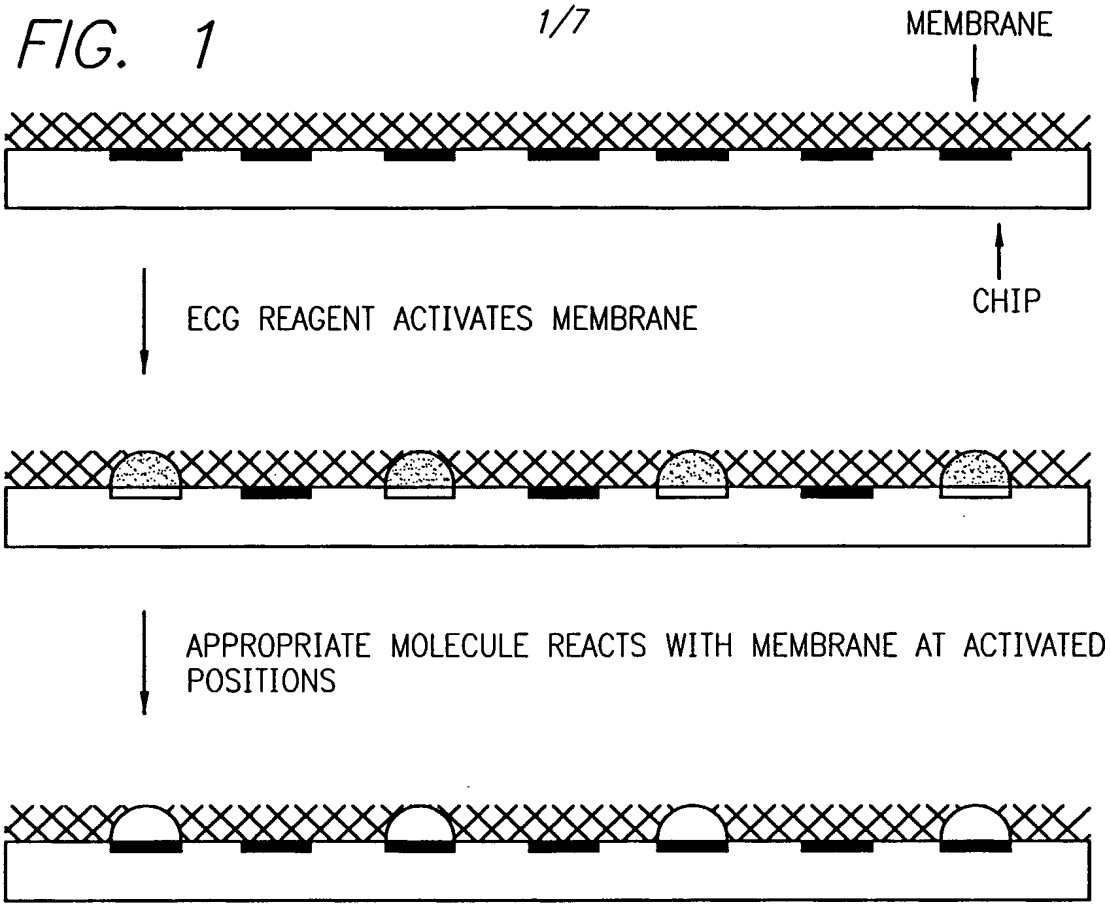
(a) placing a buffering or scavenging solution in contact with an array of electrodes that is proximate to a substrate surface, said surface being proximate to one or more acids bearing at least one protected chemical functional group attached thereto,

(b) selectively deprotecting at least one protected chemical functional group on at least one of the amino acids;

(c) bonding a second amino acid having at least one protected chemical functional group to one or more deprotected chemical functional groups of the amino acid; and

(d) repeating the selective deprotection of a chemical functional group on a bonded protected amino acid and subsequently bonding an additional amino acid to the deprotected chemical functional group until at least two separate peptides of desired length are formed on the substrate surface.

FIG. 1


MEMBRANE
CHIP
ECG REAGENT ACTIVATES MEMBRANE
APPROPRIATE MOLECULE REACTS WITH MEMBRANE AT ACTIVATED POSITIONS

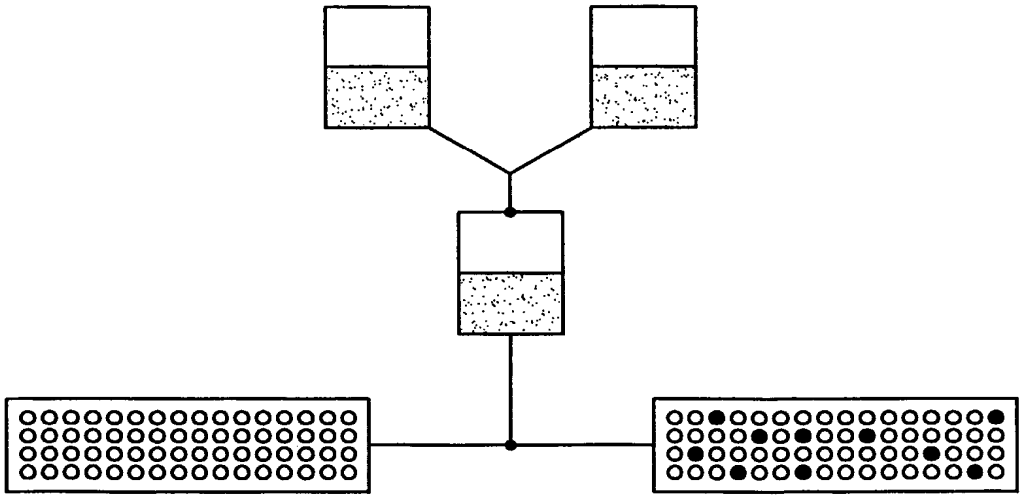


FIG 3

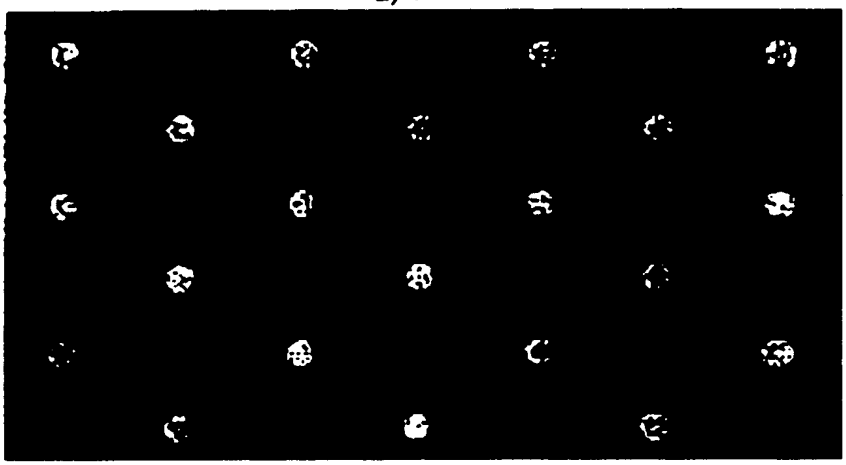


FIG. 2

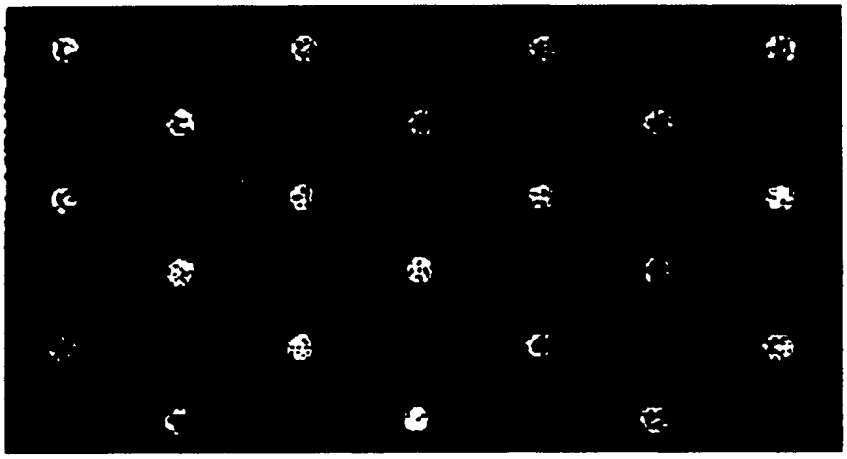


FIG. 4

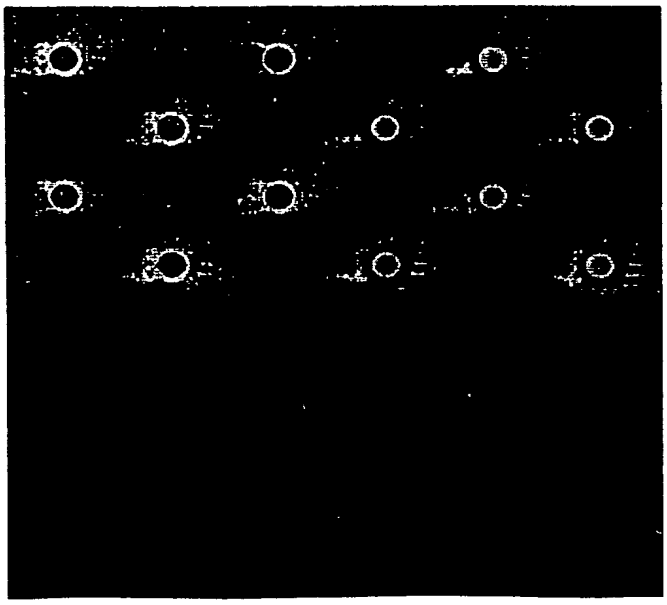


FIG. 9

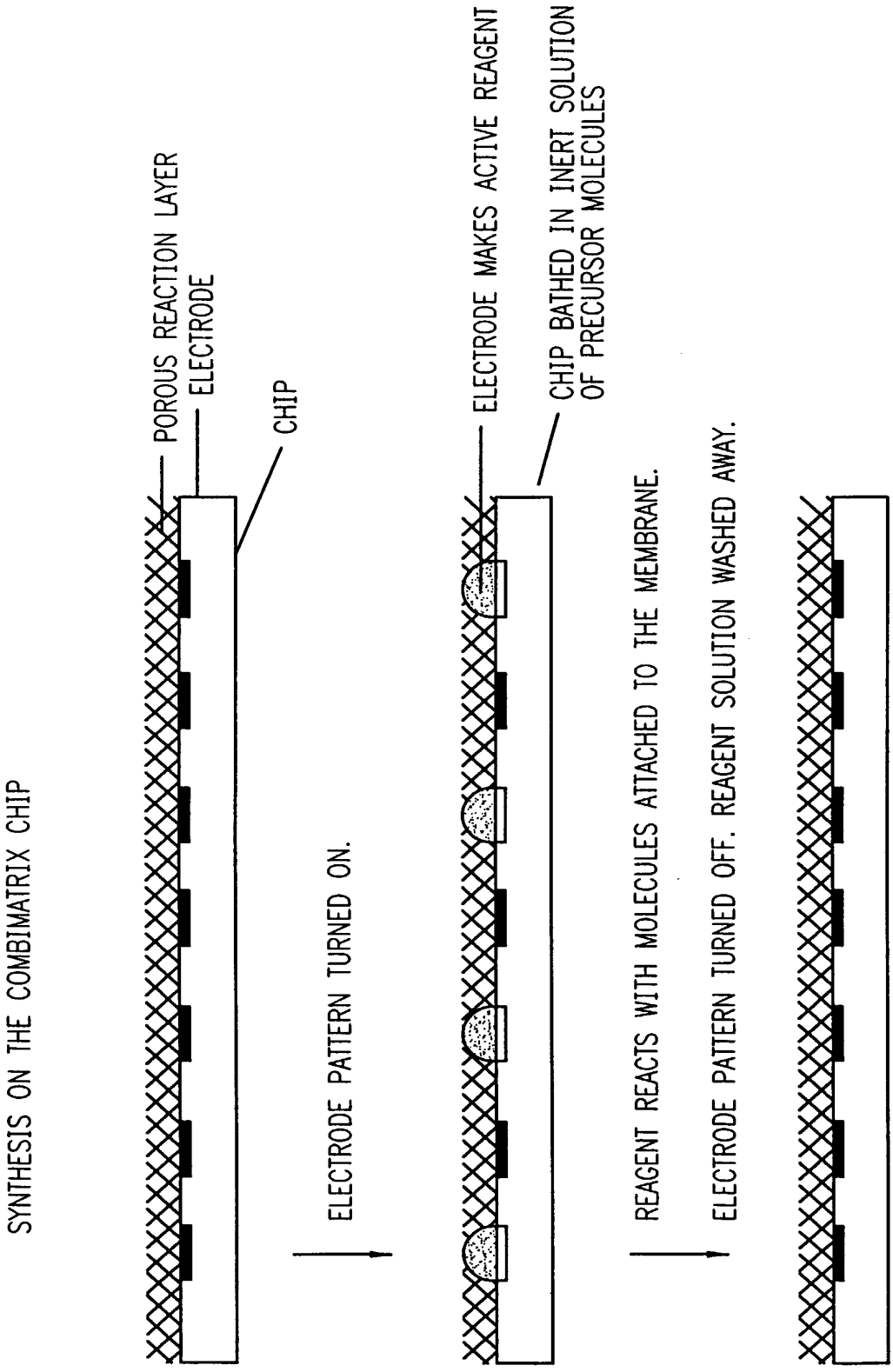

SYNTHESIS ON THE COMBIMATRIX CHIP
POROUS REACTION LAYER
ELECTRODE
CHIP
ELECTRODE PATTERN TURNED ON.
ELECTRODE MAKES ACTIVE REAGENT
CHIP BATHED IN INERT SOLUTION OF PRECURSOR MOLECULES
REAGENT REACTS WITH MOLECULES ATTACHED TO THE MEMBRANE.
ELECTRODE PATTERN TURNED OFF. REAGENT SOLUTION WASHED AWAY.

FIG. 5

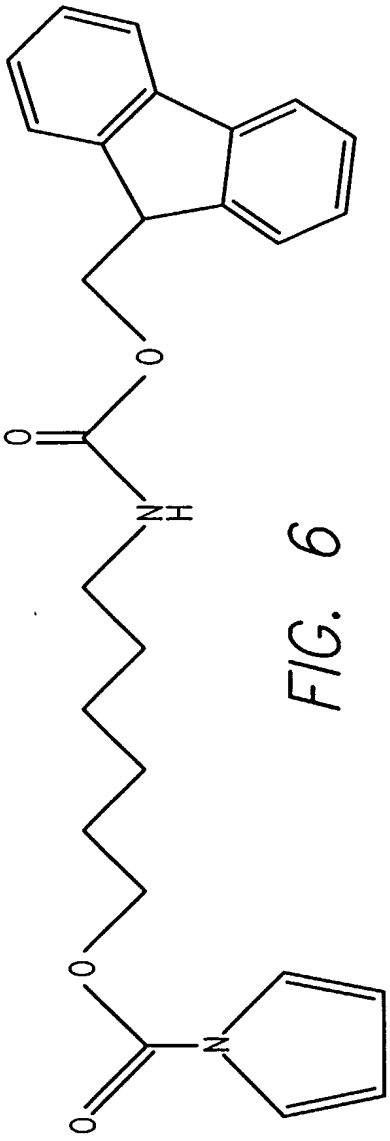


FIG. 6

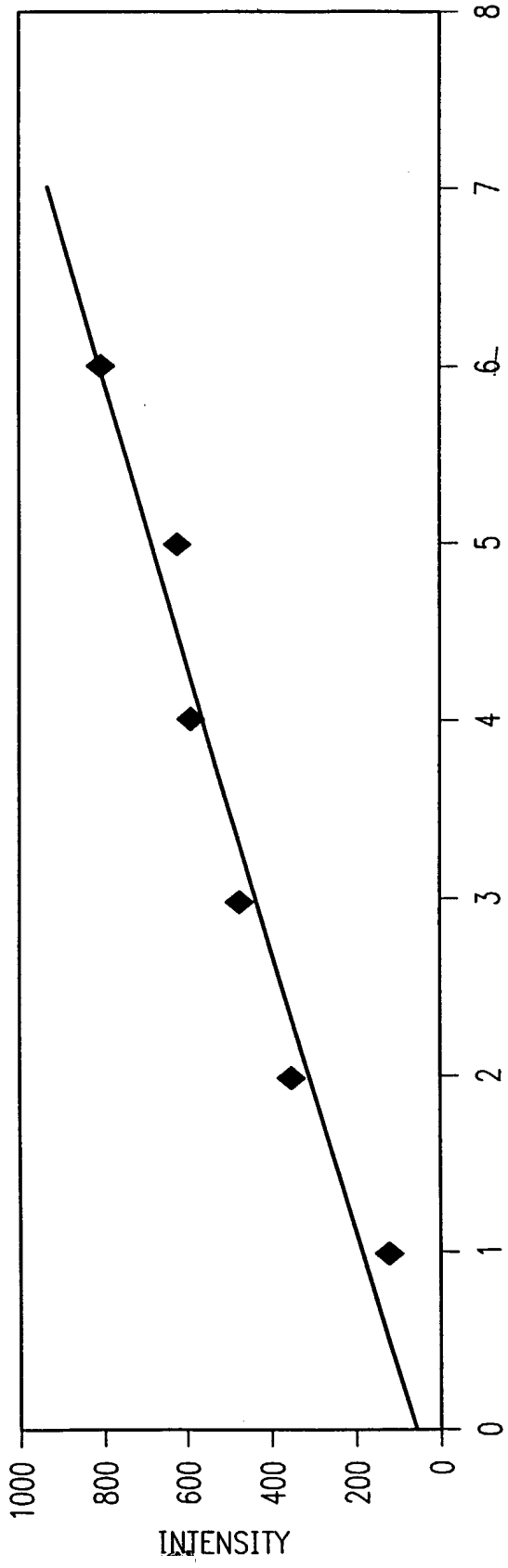


FIG. 12

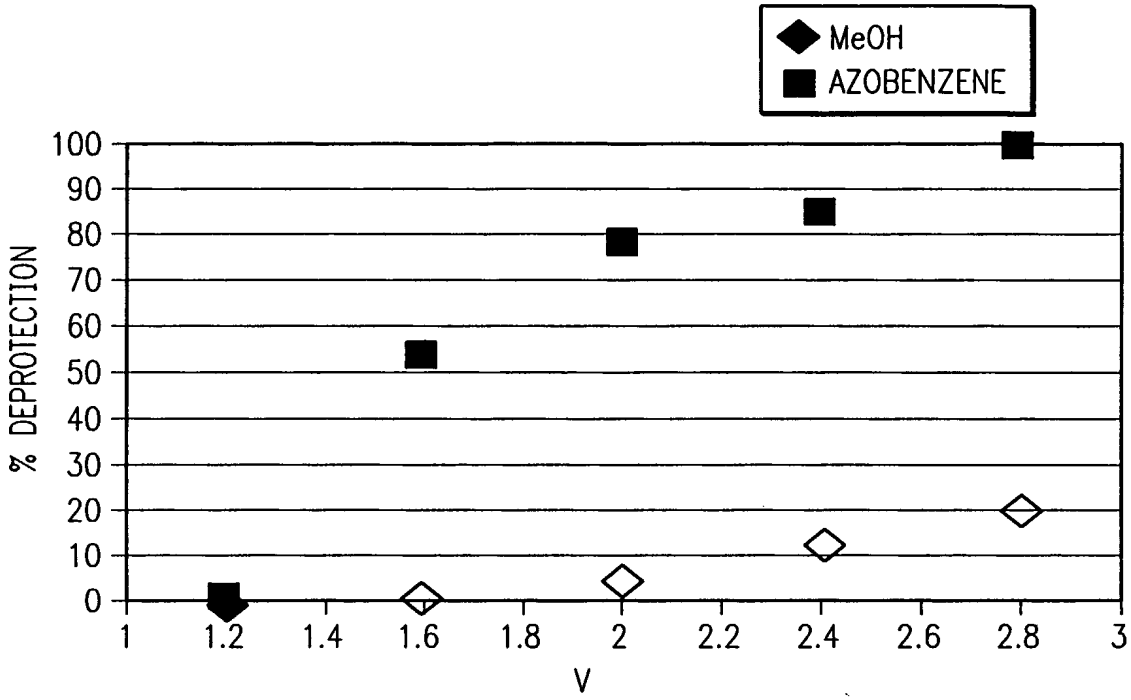

MeOH
AZOBENZENE
% DEPROTECTION
100
90
80
70
60
50
40
30
20
10
0
1
1.2
1.4
1.6
1.8
2
2.2
2.4
2.6
2.8
3
V

FIG. 7

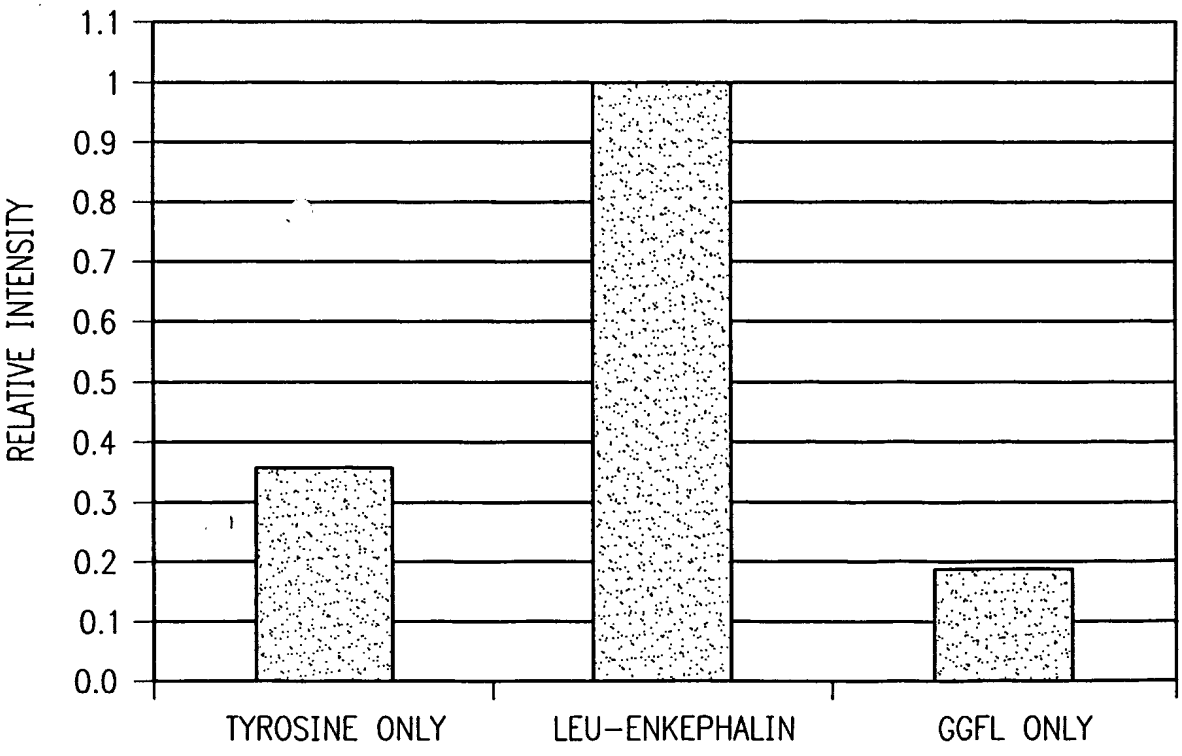

RELATIVE INTENSITY
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
TYROSINE ONLY
LEU-ENKEPHALIN
GGFL ONLY

FIG. 11

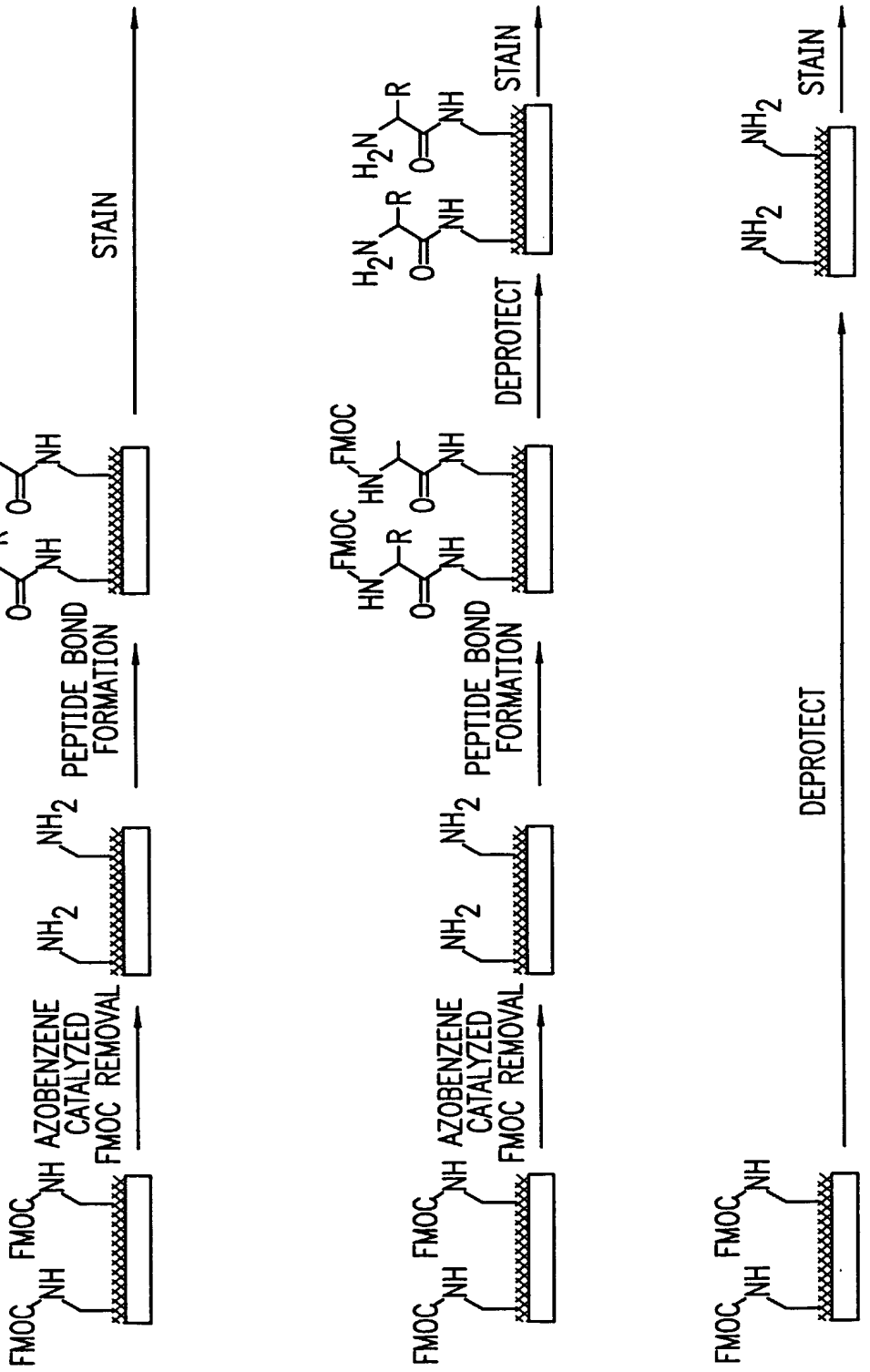

FMOC
NH
FMOC
NH
AZOBENZENE CATALYZED FMOC REMOVAL
NH2
NH2
PEPTIDE BOND FORMATION
FMOC
HN
R
O
NH
FMOC
HN
STAIN
DEPROTECT
H2N
STAIN
NH2
NH2
DEPROTECT

FIG. 8

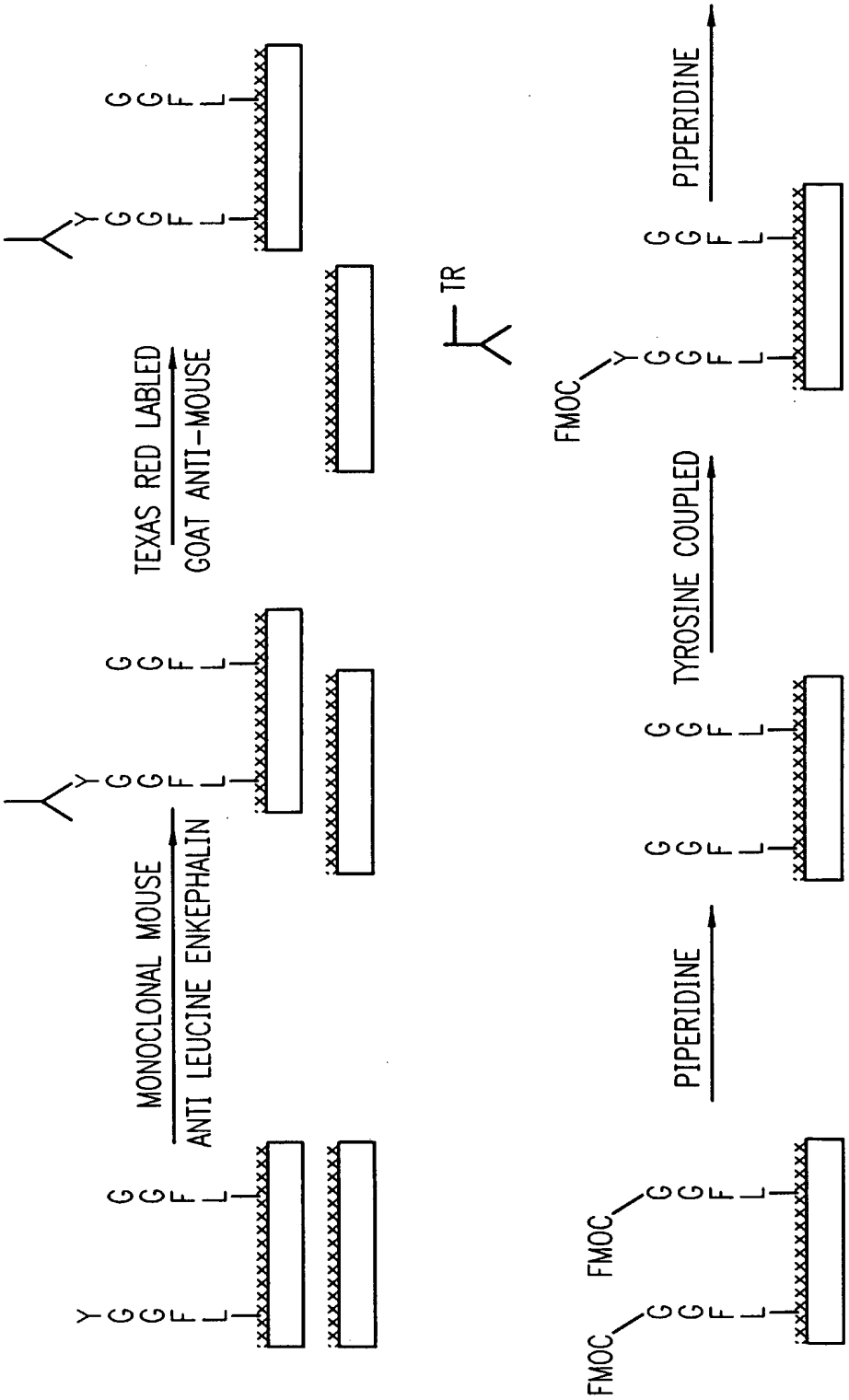

MONOCLONAL MOUSE
ANTI LEUCINE ENKEPHALIN
TEXAS RED LABLED
GOAT ANTI-MOUSE
TR
PIPERIDINE
TYROSINE COUPLED
PIPERIDINE
FMOC
FMOC
FMOC

FIG. 10

INTERNATIONAL SEARCH REPORT

al Application No
PCT/US 00/06676

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K1/04 G01N33/68 G01N33/543 B01J19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K G01N B01J

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 01221 A (COMBIMATRIX) 15 January 1998 (1998-01-15) the whole document ---	1,2,5-18
A	WO 97 49718 A (CIS BIO INTERNATIONAL) 31 December 1997 (1997-12-31) the whole document ---	1-18
X	WO 96 07917 A (NANOGEN) 14 March 1996 (1996-03-14) the whole document ---	1,2,5-18
X	WO 95 12808 A (NANOGEN) 11 May 1995 (1995-05-11) the whole document ---	1,2,5-18
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 September 2000	07/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Masturzo, P

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 22889 A (CIS BIO) 13 October 1994 (1994-10-13) claim 8	1,2
X	WO 93 22678 A (MASSACHUSETTS INSTITUTE OF TECHNOLOGY ET AL.) 11 November 1993 (1993-11-11) the whole document	1,2
P,X	WO 99 24645 A (BEN GURION UNIVERSITY OF THE NEGEV) 20 May 1999 (1999-05-20) the whole document	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

In al Application No
PCT/US 00/06676

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9801221 A	15-01-1998	AU 3588497 A	02-02-1998
		CA 2259523 A	15-01-1998
		EP 0910467 A	28-04-1999
		ZA 9705891 A	23-07-1998
WO 9749718 A	31-12-1997	FR 2750136 A	26-12-1997
		AU 3448497 A	14-01-1998
		CA 2258802 A	31-12-1997
		EP 0912593 A	06-05-1999
WO 9607917 A	14-03-1996	US 5632957 A	27-05-1997
		AU 702773 B	04-03-1999
		AU 3507095 A	27-03-1996
		BR 9508908 A	28-10-1997
		CN 1164894 A	12-11-1997
		EP 0871888 A	21-10-1998
		FI 970957 A	07-05-1997
		JP 10505497 T	02-06-1998
		US 6099803 A	08-08-2000
		US 6068818 A	30-05-2000
		US 5849486 A	15-12-1998
		US 6048690 A	11-04-2000
		US 6051380 A	18-04-2000
WO 9512808 A	11-05-1995	US 5605662 A	25-02-1997
		AU 692800 B	18-06-1998
		AU 8125794 A	23-05-1995
		AU 8522798 A	10-12-1998
		AU 8522898 A	10-12-1998
		BR 9407952 A	26-11-1996
		CA 2175483 A	11-05-1995
		CN 1141078 A	22-01-1997
		EP 0727045 A	21-08-1996
		FI 961843 A	20-06-1996
		JP 9504910 T	13-05-1997
		NZ 275962 A	28-07-1998
		US 6068818 A	30-05-2000
		US 6017696 A	25-01-2000
		US 5632957 A	27-05-1997
		US 5849486 A	15-12-1998
		US 5929208 A	27-07-1999
		US 6048690 A	11-04-2000
		US 6051380 A	18-04-2000
WO 9422889 A	13-10-1994	FR 2703359 A	07-10-1994
		AT 159028 T	15-10-1997
		DE 69406119 D	13-11-1997
		DE 69406119 T	26-03-1998
		DK 691978 T	25-05-1998
		EP 0691978 A	17-01-1996
		ES 2110228 T	01-02-1998
		GR 3025738 T	31-03-1998
		JP 8508311 T	03-09-1996
		US 5837859 A	17-11-1998
WO 9322678 A	11-11-1993	US 5846708 A	08-12-1998
		EP 0638173 A	15-02-1995
		JP 7508831 T	28-09-1995

INTERNATIONAL SEARCH REPORT

Information on patent family members

In al Application No
PCT/US 00/06676

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9322678 A		US 5653939 A	05-08-1997
WO 9924645 A	20-05-1999	AU 1518699 A	31-05-1999